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METHODS FOR THE STUDY OF PURINE METABOLISM IN THE INTACT CELL

by

© FLOYD FREDERICK SNYDER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Methods of analysis of purine metabolism in the intact cell", submitted by Floyd Frederick Snyder in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

In memory of

Frederick William

who gave and introduced to me life.

ABSTRACT

In contrast to studies with isolated enzymes most investigations of enzymes of purine ribonucleotide synthesis and interconversion in intact cells have been only qualitative or descriptive. It was the purpose of the studies reported in this thesis to develop methods by which the apparent activities of several enzymes of purine metabolism in intact cells can be measured quantitatively.

A first-order kinetic analysis was made of the reactions of purine ribonucleotide synthesis and interconversion in Ehrlich ascites tumor cells; rate constants were determined for several of these reactions by employing non-saturating purine-^{14}C base concentrations. Nucleotide monophosphate synthesis from purine bases was rate limiting for ATP and GTP synthesis.

A procedure for measuring apparent activities of individual enzymes was developed and applied to the reactions of adenine-^{14}C, hypoxanthine-^{14}C and guanine-^{14}C metabolism and the reactions of ATP-^{14}C catabolism. An analytical procedure was also developed by which drug or other effects on apparent activities of several individual enzymes can be determined in the whole cell. These procedures have been applied to the study of purine

metabolism in synchronized L5178Y cells; the uptake and conversion of purine bases to nucleotides was the rate limiting step for purine nucleoside triphosphate synthesis. An increase in enzyme activities was observed which paralleled the increase in cell volume that occurs as the cells proceed through the cycle; however, a significant decrease in adenine phosphoribosyltransferase activity was observed during the G_2-phase of the cell cycle.

The effects of actinomycin D and daunomycin on acid-soluble purine metabolism were studied; both drugs inhibited nucleotide synthesis from purine bases and inhibited IMP dehydrogenase activity more than 50% at concentrations which inhibited incorporation of bases into nucleic acids by 90%. Actinomycin D also caused increases in ATP and GTP concentrations, suggesting that these acid-soluble nucleotides may be responsible for its effects on purine nucleotide metabolism. Subsequent experiments showed that elevated ATP and GTP concentrations did inhibit certain reactions of purine metabolism in Ehrlich ascites tumor cells. Purine phosphoribosyltransferase activities were inhibited at high ATP and GTP concentrations, but whether inhibition is due to a direct effect on these enzymes, or due to inhibition of PRPP synthesis has not been determined. Elevated concentrations of ATP may have a slight inhibitory effect on AMP

synthesis from IMP, whereas high GTP concentrations severely inhibit IMP dehydrogenase activity.

The alternative routes of deoxyadenosine metabolism, i.e., deamination, phosphorylation, and cleavage, were examined in several different animal cells and tissues. All of the tissues studied exhibited a low but measurable ability to cleave the glycosidic bond of deoxyadenosine. Variations in activities of enzymes of purine metabolism were studied in Ehrlich ascites tumor cells and sublines resistant to 6-mercaptopurine and 6-methylmercaptopurine ribonucleoside, and in two lymphoma L5178Y lines which differed in their sensitivities to the delayed cytotoxic effect of 6-mercaptopurine. Several differences in apparent enzyme activities were observed.

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LIST OF ABBREVIATIONS

AMP, ADP, ATP	5'-mono-, di- and triphosphates of adenosine
dAMP, dADP, dATP	5'-mono-, di- and triphosphates of deoxyadenosine
GMP, GDP, GTP	5'-mono-, di- and triphosphates of guanosine
dGMP, dGDP, dGTP	5'-mono-, di- and triphosphates of deoxyguanosine
IMP	inosine-5'-monophosphate
XMP	xanthine-5'-monophosphate
NAD	nicotinamide adenine dinucleotide
AMPS	adenylosuccinic acid
CTP	cytidine-5'-triphosphate
UTP	uridine-5'-triphosphate
TTP	thymidine-5'-triphosphate
A	adenine
AR	adenosine
H	hypoxanthine
HR	inosine
G	guanine
GR	guanosine
X	xanthine
XR	xanthosine
UA	uric acid
PRPP	phosphoribosyl pyrophosphate
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
6MP	6-mercaptopurine
6MeMPR	6-methylmercaptopurine ribonucleoside
m, μ, n	milli, micro, nano (10^{-3}, 10^{-6}, 10^{-9})

CHAPTER 1

GENERAL INTRODUCTION

Purine derivatives and metabolites participate in reactions of many branches of intermediary metabolism and purine metabolism is closely related to cellular energy metabolism, and to DNA and RNA synthesis. The regulation of enzymes of purine nucleotide synthesis and interconversion and the relation of purine metabolism as a whole to other aspects of cellular metabolism has recently been reviewed (1,2). The many studies which have dealt quantitatively with the kinetics and regulation of enzymes of purine ribonucleotide synthesis and interconversion have also been reviewed (1,2,3,4). In contrast to studies with cell-free preparations, most investigations of enzymes of purine ribonucleotide synthesis and interconversion in intact cells have been only qualitative or descriptive.

It was the purpose of the studies reported in this thesis to develop methods by which the apparent activities of several enzymes of purine ribonucleotide synthesis, interconversion and catabolism in intact cells can be measured quantitatively. These methods rely on the use of radioactive purine precursors and the chromatographic methods of Crabtree and Henderson (5).

As a first approach, a kinetic analysis was made of the reactions of purine ribonucleotide synthesis and interconversion in Ehrlich ascites tumor cells (Chapter 2). This is followed in Chapter 3 by the basic procedure for

measuring apparent activities of individual enzymes of purine metabolism in whole cells; this procedure is expanded in Chapter 4. These procedures have then been applied to the study of: purine metabolism in synchronized cells (Chapter 5); effects of actinomycin D and daunomycin on reactions of acid-soluble purine metabolism (Chapter 6); the effects of elevated ATP and GTP concentrations on purine metabolism (Chapter 7); alternative pathways of deoxyadenosine metabolism (Chapter 8); and variations in purine metabolism among five mouse tumor sublines (Chapter 9).

The format of the following chapters is that of manuscripts and each therefore includes its own introduction, methods, results and discussion section and references; Chapter 3 has already been published (6).

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CHAPTER 2

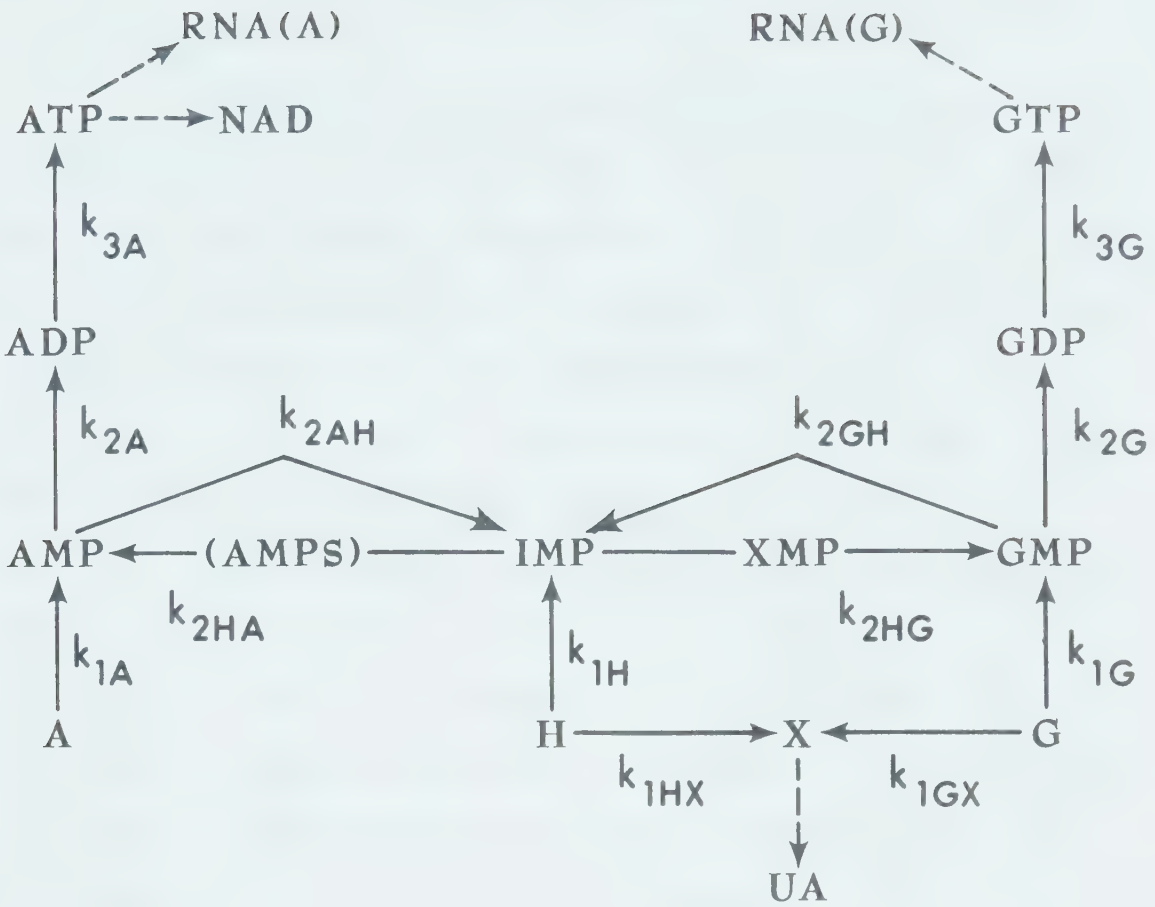
A KINETIC ANALYSIS OF PURINE NUCLEOTIDE SYNTHESIS AND INTERCONVERSION IN EHRLICH ASCITES TUMOR CELLS *IN VITRO*

INTRODUCTION

The use of radioactive precursors for the study of the kinetics of biological processes has been reviewed by Zilversmit ('60) and Robertson ('57). Kinetic analyses of biological systems may be based either on measurements of specific radioactivity of pools, or on total radioactivity measurements. Although more information may be obtained from analyses based on specific activity measurements, difficulties commonly encountered in determining total chemical pools of precursor and products, required for specific activity measurements, have caused several investigators to base their kinetic analysis on radioactivity measurements alone. Such analyses have proved useful in several applications. The kinetics of a number of biological processes have been analyzed on the basis of a two-stage consecutive first-order reaction model (A→B→C), for example, carboxytolbutamide excretion (Nelson and O'Reilly, '62), galactose metabolism in erythrocytes (Hill and Puck, '67), ^{14}C-valine efflux from chopped brain (Jones and Banks, '70), and glucose and adenine uptake into acid soluble and insoluble pools (Burns, '70). A three-stage reaction model has been developed for the kinetic analysis of galactose metabolism (Hill and Puck, '70; Hill, '71) and a three-stage first order reaction model with a branch point has been applied

to the excretion of sulfonamides (Nelson, '62).

In the present study, three and four stage consecutive irreversible reaction models with primary and secondary branch points have been applied to a first order kinetic analysis of the metabolism of purine bases in Ehrlich ascites tumor cells *in vitro*. This study has been facilitated by the present understanding of the pathways of purine metabolism (Scheme 1) and the chromatographic methods of Crabtree and Henderson ('71a) enabling the measurement of radioactivity in purine ribonucleotides, ribonucleosides and bases. By using adenine, guanine, and hypoxanthine as radioactive precursors, the kinetic parameters of the processes in Scheme 1 have been determined and used to predict the formation of ATP and GTP and their derivatives.



SCHEME 1. Pathways of purine metabolism.

MATERIALS AND METHODS

Adenine-8-^{14}C, 50 mCi/mmole; guanine-8-^{14}C, 50 mCi/mmole, and hypoxanthine-8-^{14}C, 52 mCi/mmole, were purchased from Schwarz BioResearch. Fischer's medium was purchased from Grand Island Biological Co., polyethyleneimine cellulose thin layer chromatography sheets on Mylar film from J. T. Baker Co., and cellulose thin layer chromatograms from Eastman Kodak Company.

Ehrlich ascites tumor cells were maintained in Ha/ICR mice by weekly intraperitoneal injection of 5-6 x 10^6 cells. Five or six days after implantation, cells were collected at room temperature in Fischer's medium modified to contain 25 mM phosphate buffer and without sodium bicarbonate, pH 7.4 (Crabtree and Henderson, '71a). Cells were washed three times in this medium and a 2.5% (v/v) cell suspension was made in the modified Fischer's medium. Tumor cell suspensions, 1.6 ml, and additional medium were incubated at 37° with shaking for 20 min in 10 ml Erlenmeyer flasks; a radioactive purine base was then added to make the final volume 2.0 ml, and the metabolism of the radioactive purine was measured for 90 min. Most experiments were performed at non-saturating (50 μM) concentrations of purine bases, and virtually all of the labelled precursor was consumed during the course of the experiment; other

experiments were performed at saturating (1.0 mM) purine concentrations.

At various times 100 µl of the incubation mixture was transferred to plastic tubes (10 x 75 mm, Falcon Plastics) containing 5 µl of cold 4.2 M perchloric acid, mixed and then neutralized with 5 µl of cold 4.42 N KOH. To analyze radioactivity in acid-soluble derivatives 10 µl of the supernatent was chromatographed on polyethyleneimine thin layers to separate the following ribonucleotides: GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP, NAD; or chromatographed on cellulose thin layers to separate the following bases and nucleosides: A, AR, H, HR, G, GR, X, XR, uric acid; the radioactivity in each compound was then measured. These methods have been described in detail by Crabtree and Henderson ('71a).

In experiments using adenine or guanine as labelled precursor, total acid-insoluble radioactivity was taken to be equivalent to nucleic acid adenine synthesis from adenine-^{14}C or nucleic acid guanine synthesis from guanine-^{14}C. Less than 2% of radioactive adenine nucleotides, synthesized from adenine-^{14}C, were converted to guanine nucleotides; and less than 5% of radioactive guanine nucleotides, synthesized from guanine-^{14}C, were converted to adenine nucleotides. Acid-insoluble radioactivity was measured by adding 0.5 ml of cold 0.4 M perchloric acid

to 100 µl of incubation mixture. These samples were then chilled and filtered on Whatman No. 3 filter paper discs which were successively washed with cold solutions of 2.5 ml of 0.4 M perchloric acid (twice), 2.5 ml of 0.3 M trichloroacetic acid (three times), and 2.5 ml of methanol (twice). Radioactivity on the dried filter discs was then measured (Crabtree and Henderson, '71a).

In experiments using hypoxanthine as precursor, substantial amounts of radioactivity were found in both nucleic acid adenine and nucleic acid guanine. In this case 100 µl of incubation mixtures was transferred to 1.0 ml glass ampules containing 5 µl of 4.2 M perchloric acid, the acid insoluble precipitate was washed four times with 1.0 ml of 0.4 M perchloric acid, dried in vacuo, and to each ampule 100 µl of 1.0 N HCl was added. The ampules were sealed and placed in a boiling water bath for 60 min to hydrolyze the nucleic acids. Portions of the hydrolysates (50 µl) were chromatographed on Whatman No. 1 paper with carrier adenine and guanine for 5.5 hr (descending) with methanol, formic acid and water (70:15:5). Adenine and guanine spots having R_f values of 0.61 and 0.43 respectively, were cut out and their radioactivity measured.

Chromatography of the acid-soluble extracts was also performed on a Varian-Aerograph LCS-1000 liquid chromatograph utilizing 20 µl of extract per analysis.

Acid-soluble nucleotides were separated utilizing a linear gradient of potassium phosphate and potassium chloride at pH 4.5, as described by Brown ('70). Amounts of individual nucleotides were determined by comparing the peak areas, measured by planimeter, with a series of standards of commercial nucleotides.

ANALYSIS AND RESULTS

Non-saturating precursor concentration: kinetic analysis

Crabtree ('70) has shown that nucleotide synthesis in Ehrlich ascites tumor cells is proportional to adenine or guanine concentrations from 1 to 100 μM and also to hypoxanthine concentration from 1 to 50 μM, nucleotide synthesis becoming saturated between 50 and 100 μM hypoxanthine. In the present study it has been shown that nucleotide synthesis from hypoxanthine in Ehrlich ascites tumor cells was saturated between 60 and 70 μM hypoxanthine (Fig. 1). A concentration of 50 μM adenine, guanine and hypoxanthine was chosen, so that the precursor concentration was less than the saturation level for base uptake by the cell and conversion to nucleotide; thus the proportionality between rate of nucleotide synthesis and substrate concentration was maintained throughout the experiment. Although

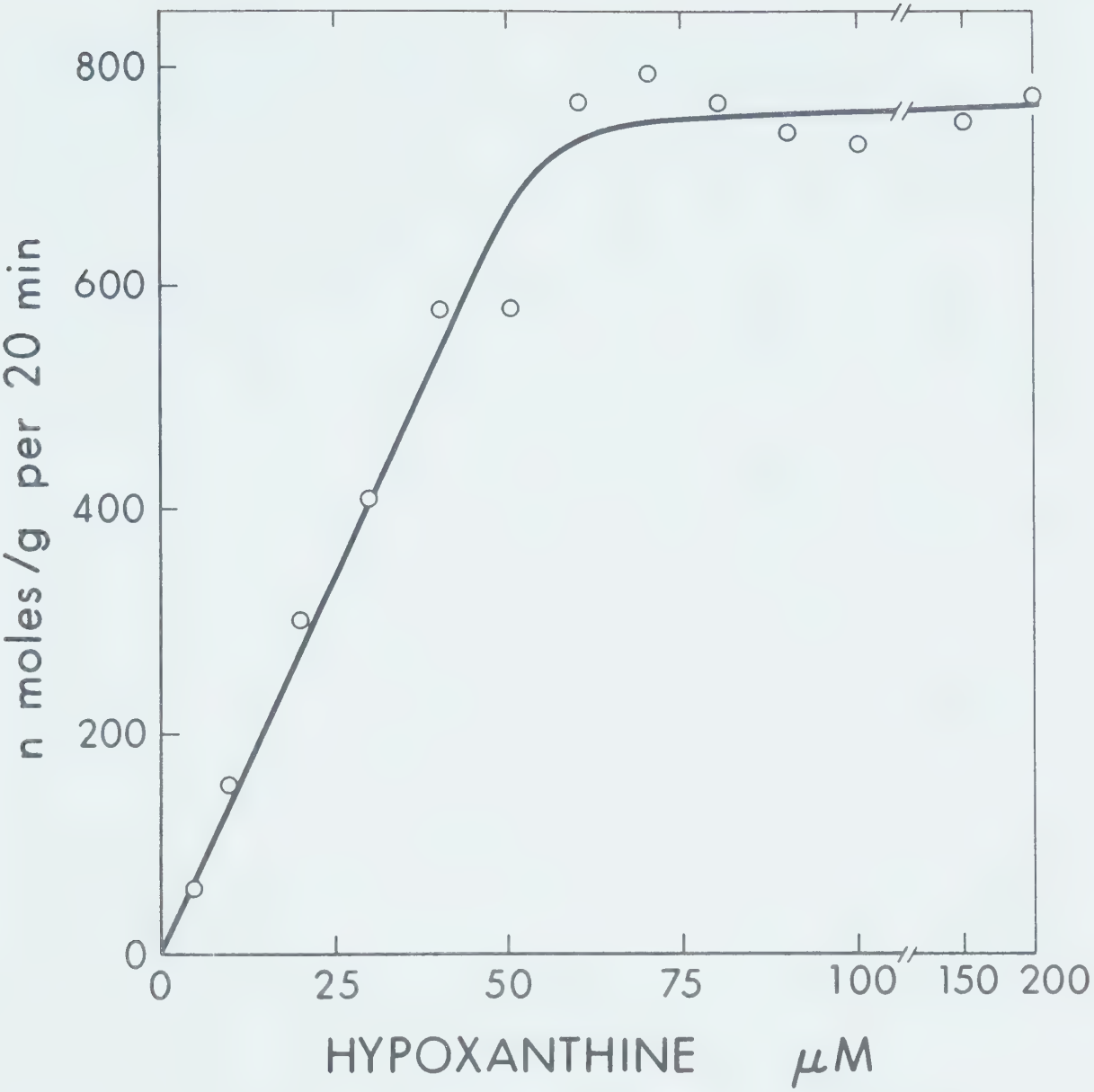


Figure 1. Acid soluble nucleotide synthesis from hypoxanthine ^{14}C.

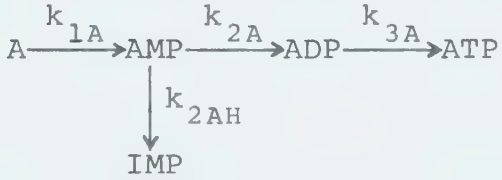
Incubation time: 20 min

there may be a small amount of endogenous purine base formation during the course of an experiment (Crabtree and Henderson, '71b), free purine bases are not detectable in Ehrlich ascites tumor cells (Murray, '66). Thus a kinetic model in which the specific activity of the precursor is constant is applicable to this study of purine nucleotide synthesis and interconversion.

The sum of the total amount of radioactivity in precursor and products was measured and remained constant throughout these experiments and the kinetic analysis is based on the conservation of the total radioactivity in the system. The conversion of purine bases to nucleoside triphosphates is proposed to follow a first order consecutive reaction with appropriate branch points. A set of differential equations may be written for each of the reactions represented in Schemes 2, 3, and 4, and these equations may be solved by the operator method of Rodiguin and Rodiguina ('64, p. 109). First order rate constants were determined from the rate of utilization of purine bases for nucleotide synthesis and the observed steady state concentrations of nucleoside mono- and diphosphates. The consecutive first order model was used to predict the rates of formation of the radioactive nucleoside triphosphates, ATP and GTP, and their derivatives, nucleic acid adenine and nucleic acid guanine.

Adenine-^{14}C as precursor

The conversion of radioactive adenine to purine nucleotides was measured for 90 min; every 5 or 10 min samples were taken and the radioactivity in individual purine ribonucleotides, ribonucleosides, and bases was determined. These transformations are proposed to follow a first order consecutive three-stage reaction with a branch point from the first product, AMP (Scheme 2).



SCHEME 2

The rate of utilization of adenine-^{14}C followed the first order relationship $-dA/dt = k_{1A}A$, the solution to which is $A=A^{\circ}e^{-(k_{1A})t}$, where A° is the initial concentration of adenine and A is the concentration of adenine at time t. The slope of the plot of log A versus time (Fig. 2) gave $k_{1A}=0.0596\pm0.0057\ min^{-1}$ (mean ± average deviation from the mean).

The second step rate constants k_{2A} and k_{2AH} were determined from the following two relationships:

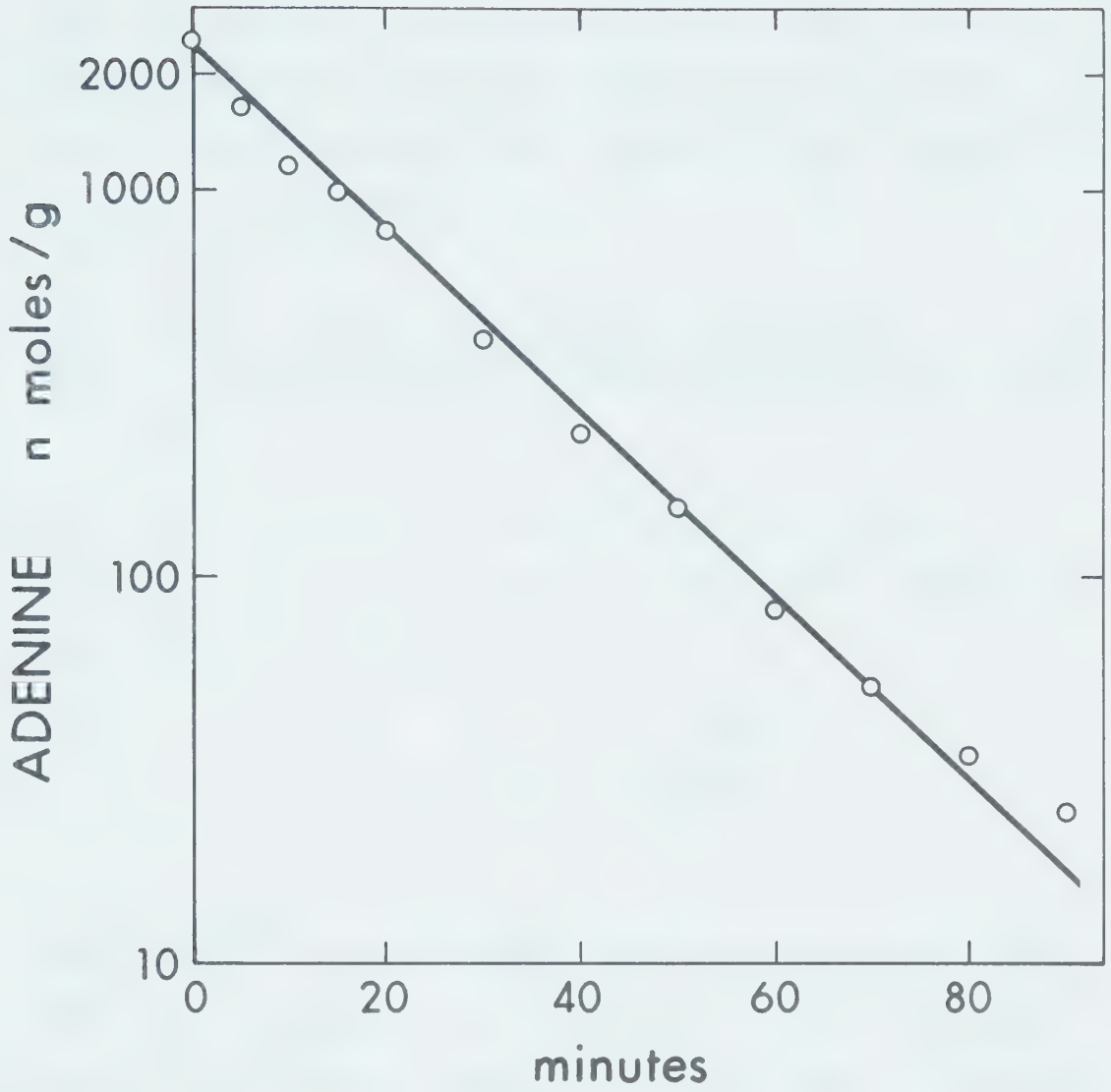


Figure 2. Semi-log plot of adenine-^{14}C utilization.
Initial precursor concentration: 50 µM

(i) The ratio k_{2A}/k_{2AH}, represented by the sum of radioactive products along either branch of the pathway, should remain constant with respect to time and may therefore be represented by:

$$[1] \quad \frac{k_{2A}}{k_{2AH}} = \left[\frac{\text{ADP+ATP+NAD+Nucleic acid adenine}}{\text{IMP+XMP+GMP+GDP+GTP+HR+H+GR+G+XR+X+Uric Acid}}\right]^t$$

(ii) The rate of formation of AMP is given by $dAMP/dt = k_{1A}A - k_{2A}AMP - k_{2AH}AMP$ and at steady state concentrations of AMP, where $dAMP/dt = 0$,

$$[2] \quad k_{2A} + k_{2AH} = \frac{k_{1A}A^{mAMP}}{AMP^{mAMP}}$$

where AMP^{mAMP} is the maximum concentration of AMP and A^{mAMP} the concentration of adenine when AMP initially attains its maximum value, after the method of Rodiguin and Rodiguina ('64, p. 87).

The radioactive AMP pool reached a steady state value of 7 nmoles/g after 5 min of incubation (Fig. 3), at which time the labelled adenine concentration was 1640 nmoles/g. The ratio k_{2A}/k_{2AH} was 68±13 over the 90 min course of the experiment. Application of equations [1] and [2] gave $k_{2A} = 13.9 \pm 1.4$ min^{-1} and $k_{2AH} = 0.20 \pm 0.06$ min^{-1}.

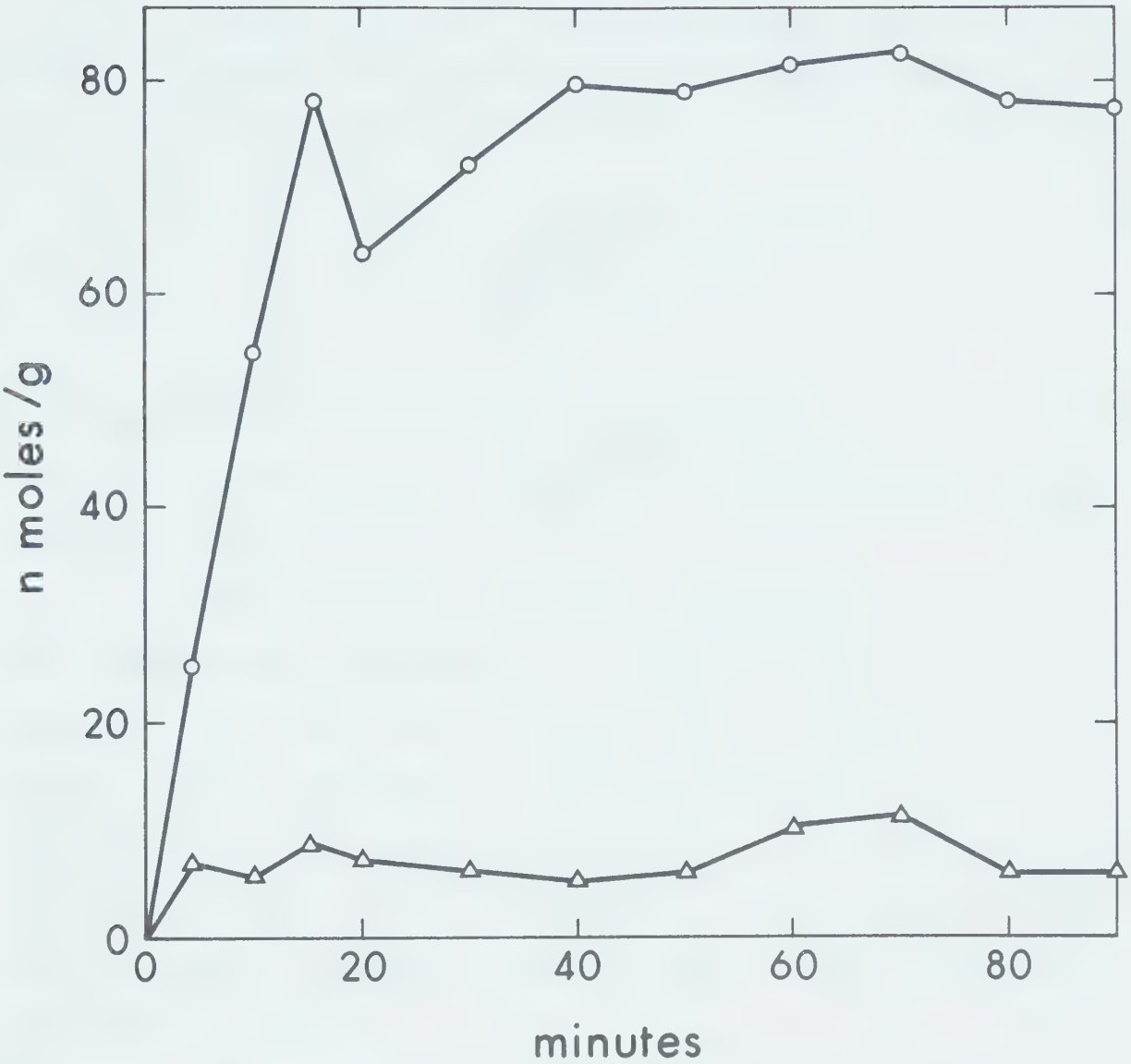


Figure 3. Concentrations of radioactive AMP and ADP.

Precursor: adenine-^{14}C, 50 μM initial concentration

(△) AMP
(○) ADP

The formation and further phosphorylation of ADP may be represented by $dADP/dt = k_{2A}AMP - k_{3A}ADP$, and at the steady state, $dADP/dt = 0$, or

$$[3] \qquad k_{3A} = \frac{k_{2A}AMP^{mADP}}{ADP^{mADP}}$$

where AMP^{mADP} is the concentration of AMP when ADP initially attains its maximum value and ADP^{mADP} is the maximum concentration of ADP.

The third step constant k_{3A} was calculated from the steady state concentration of radioactive ADP, 77.6 nmoles/g, which was attained after 15 min incubation at which time the concentration of radioactive AMP was 8.9 nmoles/g (Fig. 3). Substitution of these values into equation 3, gave $k_{3A} = 1.62 \pm 0.16$ min^{-1}. The solution to all rate constants of Scheme 2 has thus been obtained and these are given in Table 1.

Application of the transformed function method of Rodiguin and Rodiguina ('64, p. 109) to the set of differential equations defining Scheme 1, gave equation [4]. The formation of labelled ATP and its products is predicted by equation [4] if the rate constants of Table 1 and the initial concentration of adenine, $A^{\circ} = 2414$ nmoles/g, are substituted into equation [4]. The theoretical curve for

Table 1. Rate constants of adenine metabolism

Reaction	Rate Constant (min^{-1})
A⟶AMP	$k_{1A} = 0.0596 \pm 0.0057$
AMP⟶ADP	$k_{2A} = 13.9 \pm 1.4$
AMP⟶IMP	$k_{2AH} = 0.20 \pm 0.06$
ADP⟶ATP	$k_{3A} = 1.62 \pm 0.16$

EQUATION [4]

$$ATP = A^{o}\left[\frac{k_{2A}}{k_{2A}+k_{2AH}} - \frac{k_{2A}k_{3A}}{(k_{2A}+k_{2AH}-k_{1A})(k_{3A}-k_{1A})}\ e^{-(k_{1A})t}\right.$$

$$- \frac{k_{1A}k_{2A}k_{3A}}{(k_{2A}+k_{2AH})(k_{1A}-k_{2A}-k_{2AH})(k_{3A}-k_{2A}-k_{2AH})}\ e^{-(k_{2A}+k_{2AH})t}$$

$$\left. - \frac{k_{1A}k_{2A}}{(k_{1A}-k_{3A})(k_{2A}+k_{2AH}-k_{3A})}\ e^{-(k_{3A})t}\right]$$

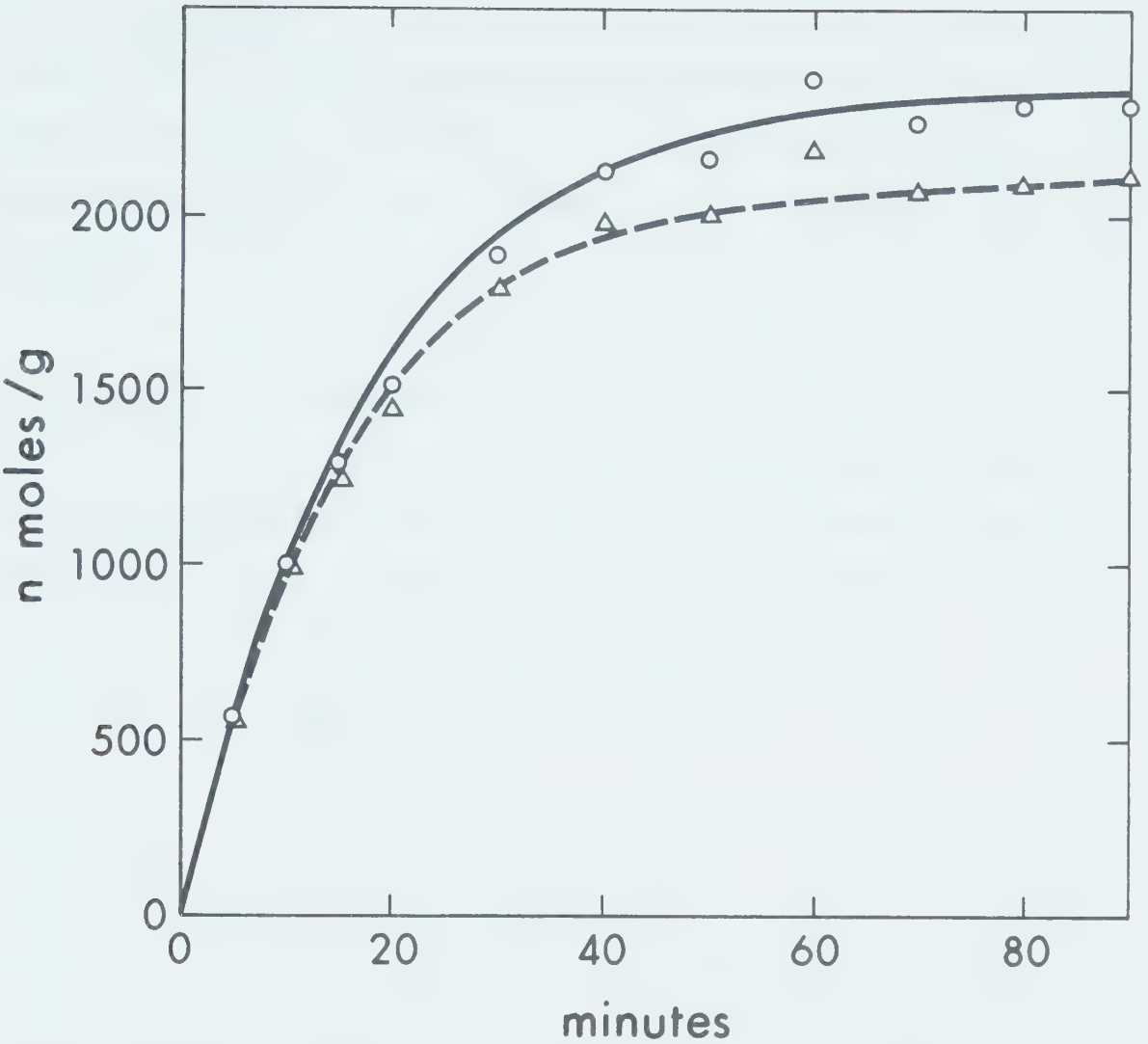


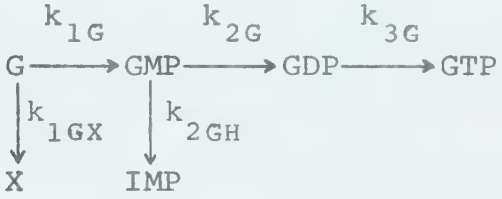
Figure 4. Concentration of radioactive ATP and of ATP plus nucleic acid adenine.

Precursor: adenine-^{14}C, 50 μM initial concentration.
Solid curve was calculated from rate constants in Table 1.
(Δ) ATP (dashed curve)
(o) ATP plus nucleic acid adenine

ATP formation predicted by this model has been plotted in Figure 4 together with experimental measurements of radioactive ATP and also the sum of labelled ATP, acid insoluble adenine and NAD, which are formed from ATP. The latter measurements are in good agreement with the theoretical curve.

Guanine-^{14}C as precursor

A first order three-stage consecutive reaction with primary and secondary branch points (Scheme 3) is advanced as a model for the metabolism of guanine.



SCHEME 3

From the rate of decrease in labelled guanine, the first branch point rate constants k_{1G} and k_{1GX} were determined. The rate of guanine utilization is given by $-dG/dt = G^{o}(k_{1G} + k_{1GX})$, the solution of which is $G = G^{o}e^{-(a_{1G})t}$, where G is the concentration of guanine at time t, G^{o} is the initial concentration of guanine, and $a_{1G} = k_{1G} + k_{1GX}$. From the plot of log G versus time (Fig. 5) it may be seen

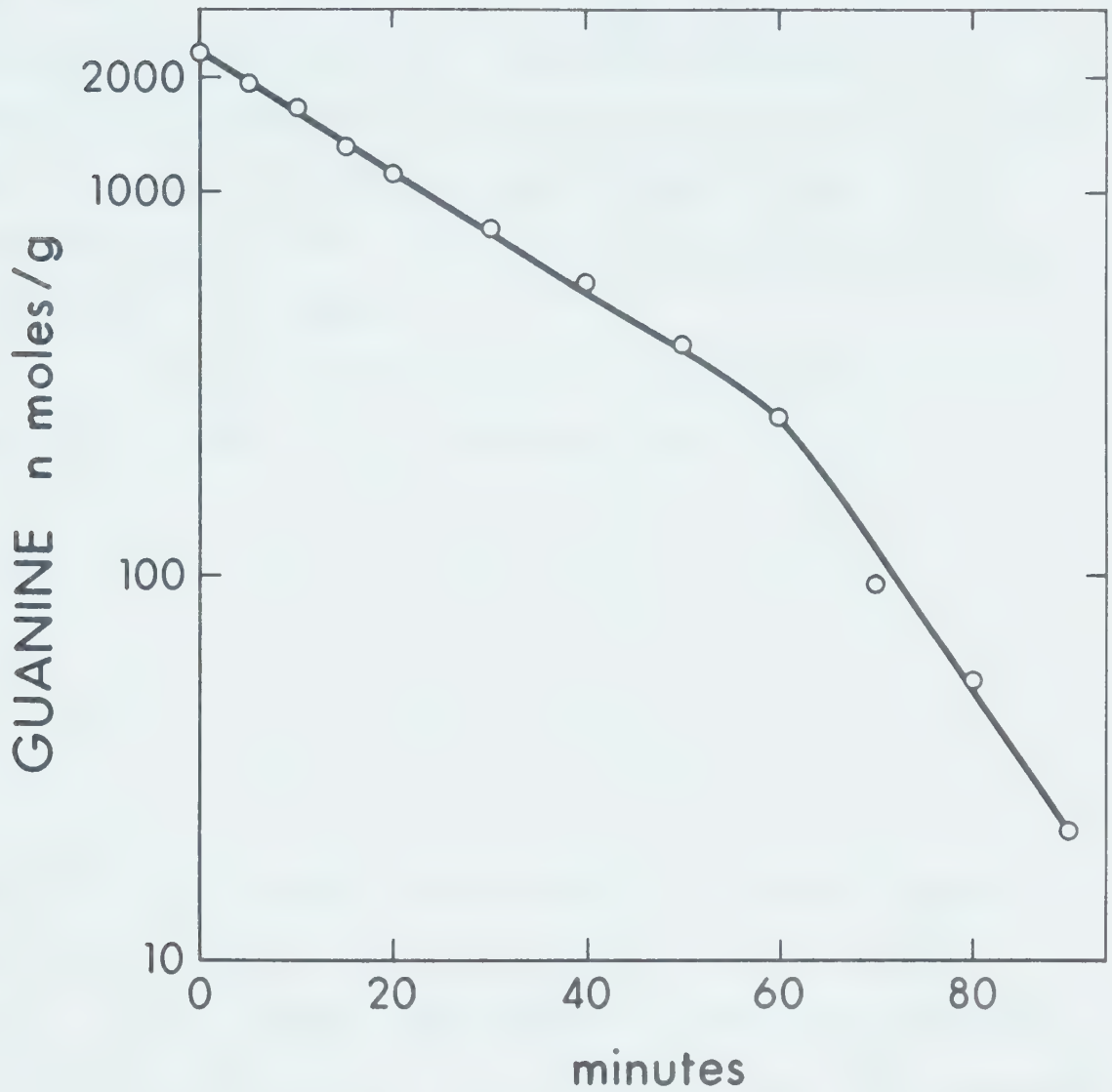


Figure 5. Semi-log plot of guanine-^{14}C utilization.
Initial precursor concentration: 50 µM.

that the plot is linear, but biphasic, having a different slope over the last 30 min. Eighty-six percent of the labelled guanine was utilized in the first 60 min, and the rate of guanine utilization over this period was used to establish $a_{1G} = 0.0357 \pm 0.0017\ min^{-1}$. From 60 to 90 min, $a_{1G} = 0.0877 \pm 0.0086\ min^{-1}$.

The rate of formation of xanthine and uric acid is given by $dX/dt = k_{1GX}G$, which has the solution:

$$X = k_{1GX}G^{o}\left[\frac{1}{a_{1G}} - \frac{1}{a_{1G}} e^{-(a_{1G})t}\right]$$

[5] or $$k_{1GX} = \frac{X}{G^{o}}\left[\frac{a_{1G}}{1 - e^{-(a_{1G})t}}\right]$$

where G^{o} is the initial concentration of labelled guanine and X is the concentration of xanthine plus uric acid at time t. Table 2 gives experimentally determined values for X, from which the constant k_{1GX} was calculated using equation [5] to be $0.0056 \pm 0.0008\ min^{-1}$ over the initial 70 min of the course. Since $a_{1G} = 0.0357\ min^{-1}$, k_{1G} is therefore $0.0301 \pm 0.0025\ min^{-1}$.

The second stage rate constants k_{2G} and k_{2GH} were evaluated in a manner similar to the evaluation of the rate constants k_{2A} and k_{2AH}. Thus the two applicable equations are:

Table 2. Xanthine and Uric acid formation from guanine and hypoxanthine*

Time (min)	Guanine as precursor	Hypoxanthine as precursor
	Xanthine plus Uric Acid (nmoles/g)	
5	56	4.0
10	87	7.8
15	119	13.3
20	157	20.2
30	223	26.0
40	278	40.9
50	329	45.1
60	386	41.9
70	448	44.1
80	465	41.5
90	436	49.5

* Cells were incubated with 50 μM guanine-^{14}C or hypoxanthine-^{14}C and radioactivity in products measured at various times.

$$[6] \qquad \frac{k_{2G}}{k_{2GH}} = \left[\frac{\text{GDP+GTP+Nucleic acid guanine}}{\text{IMP+AMP+ADP+ATP+NAD+H+HR+A+AR}}\right]^{t}$$

$$[7] \qquad k_{2G} + k_{2GH} = \frac{k_{1G}G^{mGMP}}{GMP^{mGMP}}$$

where G^{mGMP} is the concentration of guanine at the time when GMP initially attains its maximum value, and GMP^{mGMP} is the maximum value of GMP. The ratio k_{2G}/k_{2GH} was found to be 33 ± 6 over the first 60 min and 16.9 ± 0.7 from 60 to 90 min. A value of 6.4 nmoles/g, attained after 10 min incubation, was chosen as the maximum concentration of radioactive GMP for the purpose of these calculations*; at this time the labelled guanine concentration was 1652 nmoles/g. Substitution of these values in equations [6] and [7] gave k_{2G} = 7.54 ± 0.62 min^{-1} and k_{2GH} = 0.23 ± 0.06 min^{-1}.

The third step constant k_{3G} was obtained from the steady state value of GDP, 30.6 nmoles/g, at 30 min, at which time the labelled GMP concentration was 9.1 nmoles/g (Fig. 6), and equation [8], which is analogous to equation [3].

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* The earliest maximum in GMP (10 min) and GDP (30 min) concentrations (Fig. 6) were taken as approximate steady state concentrations for the calculation of rate constants; although there was considerable fluctuation in the concentrations of these nucleotides, their mole percent of the total labelled purines remained relatively constant.

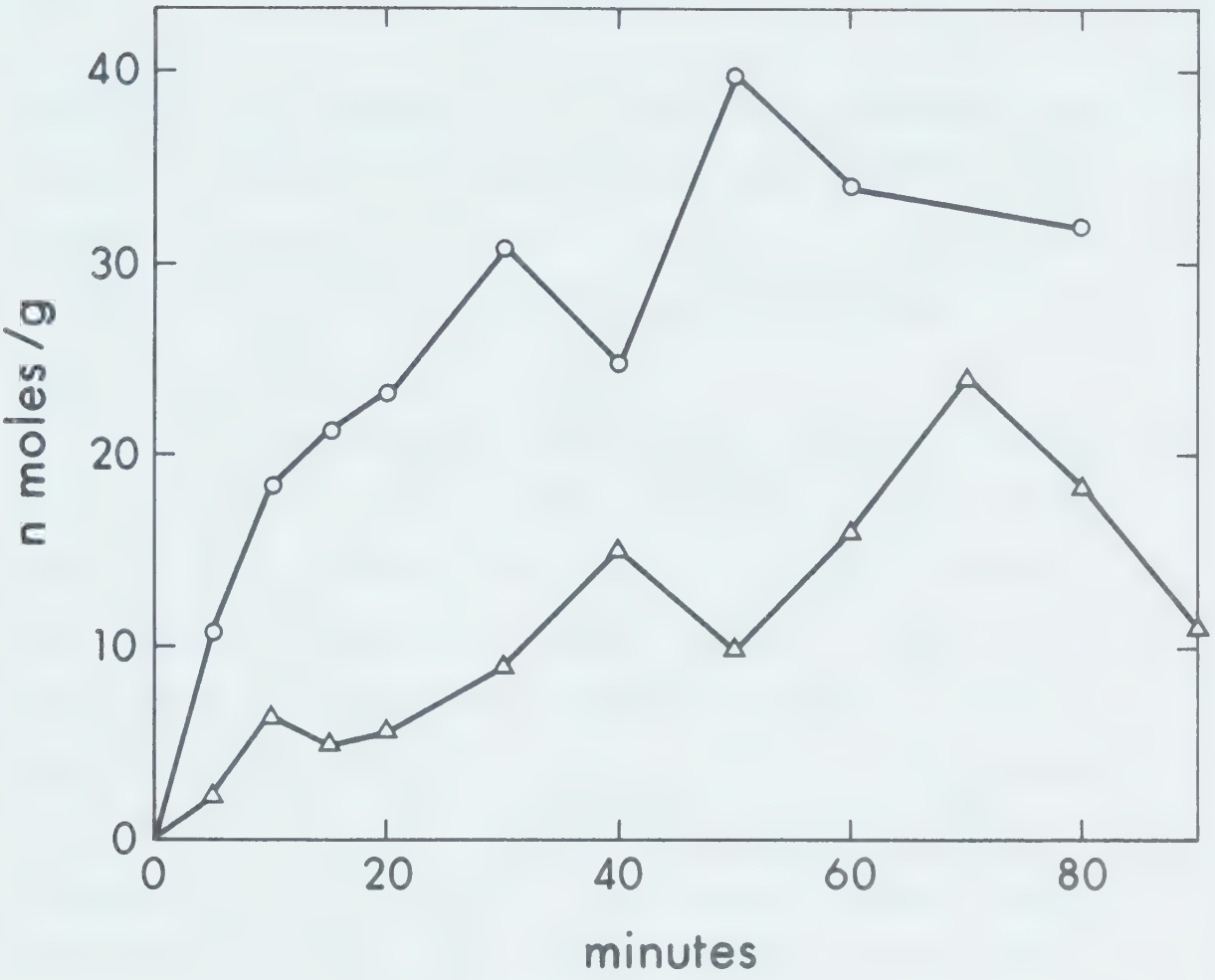


Figure 6. Concentrations of radioactive GMP and GDP.

Precursor: guanine-^{14}C, 50 μM initial concentration.

(Δ) GMP
(o) GDP

$$[8] \qquad k_{3G} = \frac{k_{2G}GMP^{mGDP}}{GDP^{mGDP}}$$

GMP^{mGDP} is the concentration of GMP at the time when GDP initially attains its maximum value, and GDP^{mGDP} is the maximum concentration of GDP. Solution of equation [8] gave $k_{3G} = 2.24 \pm 0.18$ min^{-1}. The rate constants for Scheme 3 are tabulated in Table 3.

By solving the differential equations defining the reactions of Scheme 3, the following solution (equation [9]) was obtained for GTP formation from guanine. Substitution of the rate constants given in Table 3 and the initial concentration of labelled guanine, G^{o}=2168 nmoles/g, into equation [9] gave the plot of GTP formation shown in Figure 7. The experimentally measured GTP accumulation over 90 min is also shown, together with the values for GTP plus acid insoluble guanine; the latter measurements are in good agreement with the theoretical curve.

Hypoxanthine-8-^{14}C as precursor

The model proposed for hypoxanthine metabolism is a first order consecutive four-stage reaction with primary and secondary branch points (Scheme 4).

Table 3. Rate constants of guanine metabolism

Reaction	Rate Constant (min^{-1})
G ⟶ GMP	$k_{1G} = 0.0301 \pm 0.0025$
G ⟶ X	$k_{1GX} = 0.0056 \pm 0.0008$
GMP ⟶ GDP	$k_{2G} = 7.54 \pm 0.62$
GMP ⟶ IMP	$k_{2GH} = 0.23 \pm 0.06$
GDP ⟶ GTP	$k_{3G} = 2.24 \pm 0.18$

EQUATION [9]

$$GTP = G^{\circ}\left[\frac{k_{1G}k_{2G}}{(k_{1G}+k_{1GX})(k_{2G}+k_{2GH})}\right.$$

$$- \frac{k_{1G}k_{2G}k_{3G}}{(k_{1G}+k_{1GX})(k_{2G}+k_{2GH}-k_{1G}-k_{1GX})(k_{3G}-k_{1G}-k_{1GX})} \; e^{-(k_{1G}+k_{1GX})t}$$

$$- \frac{k_{1G}k_{2G}k_{3G}}{(k_{2G}+k_{2GH})(k_{1G}+k_{1GX}-k_{2G}-k_{2GH})(k_{3G}-k_{2G}-k_{2GH})} \; e^{-(k_{2G}+k_{2GH})t}$$

$$\left. - \frac{k_{1G}k_{2G}}{(k_{1G}+k_{2GX}-k_{3G})(k_{2G}+k_{2GH}-k_{3G})} \; e^{-(k_{3G})t}\right]$$

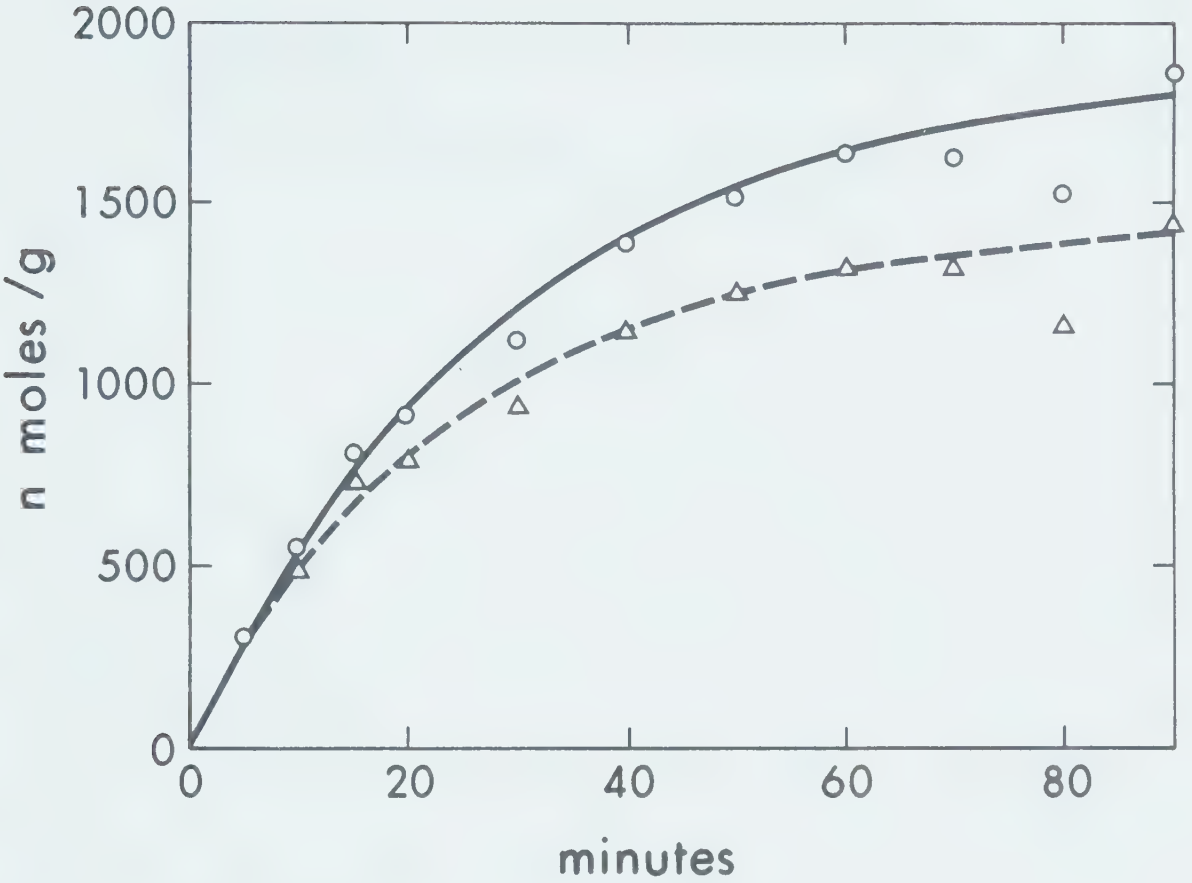
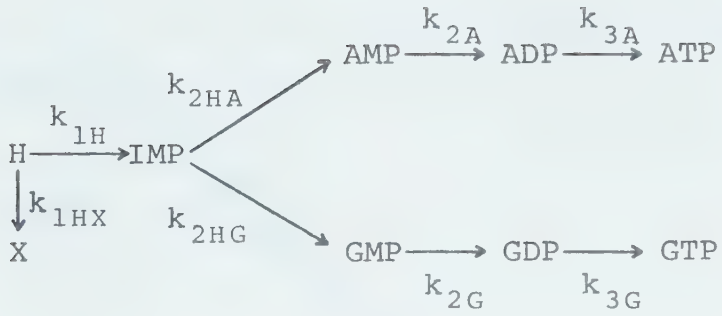


Figure 7. Concentration of radioactive GTP and of GTP plus nucleic acid guanine.

Precursor: guanine-^{14}C, 50 μM initial concentration.
Solid curve was calculated from rate constants in Table 3.
(Δ) GTP (dashed curve)
(o) GTP plus nucleic acid guanine



SCHEME 4

The conversion of hypoxanthine to other products is given by $-dH/dt = (k_{1H} + k_{1HX})H^{\circ}$, the solution of which is $H = H^{\circ}e^{-(a_{1H})t}$, where H is the concentration of the precursor hypoxanthine at time t, H° is the initial concentration of hypoxanthine, and $a_{1H} = k_{1H} + k_{1HX}$. From measurements of the rate of utilization of radioactive hypoxanthine, (Fig. 8), the constant a_{1H} was found to be 0.0543 ± 0.0046 min^{-1}. The constant k_{1HX} was evaluated by substituting values for xanthine and uric acid formation at various time intervals (Table 2) into equation [10] which is analogous to equation [5]:

[10] $$k_{1HX} = \frac{X}{H^{\circ}} \left[\frac{a_{1H}}{1 - e^{-(a_{1H})t}} \right]$$

where X is the sum of uric acid and xanthine concentrations at time t, and H° is the initial concentration of hypoxanthine,

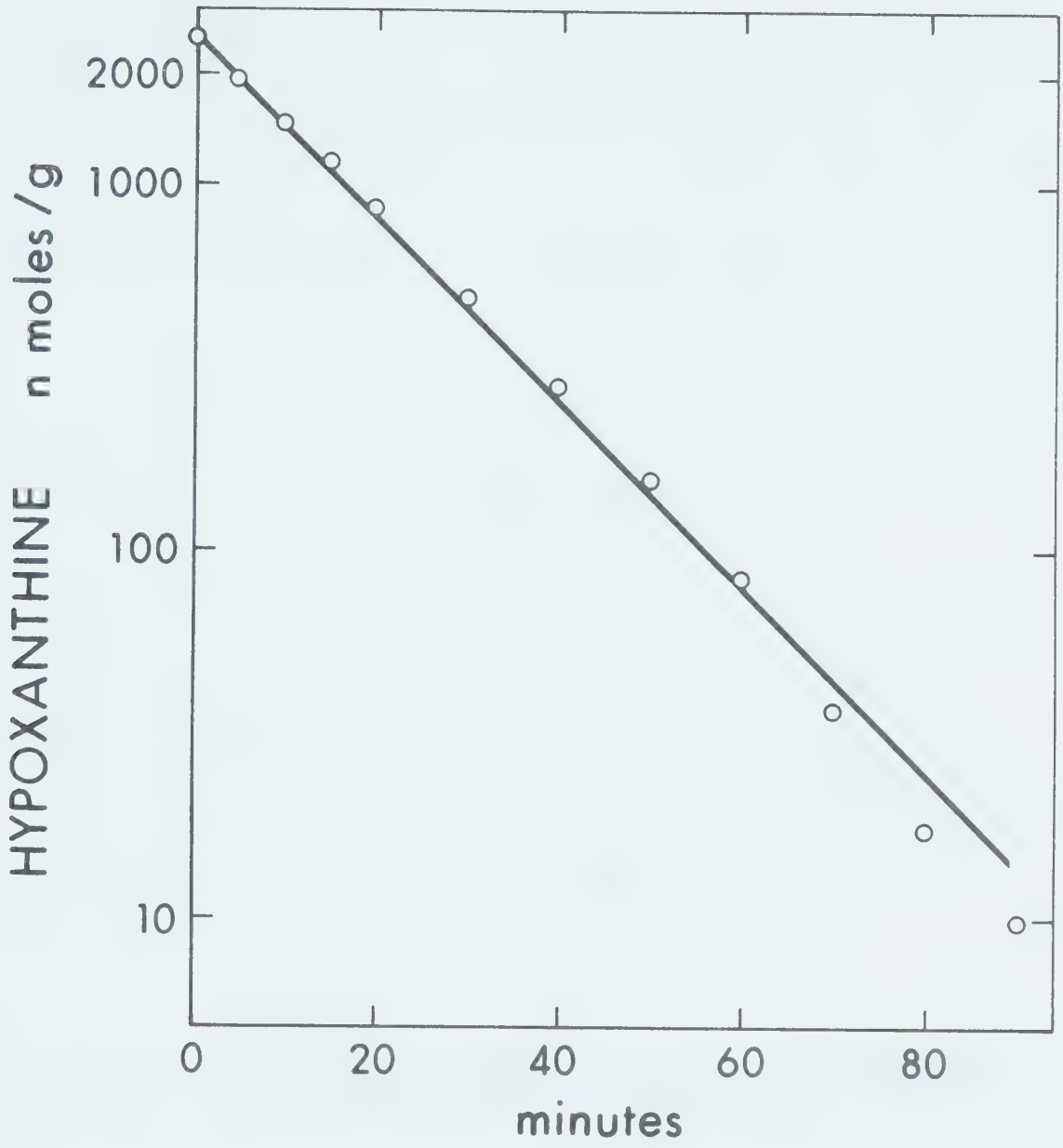


Figure 8. Semi-log plot of hypoxanthine-^{14}C utilization
Initial precursor concentration: 50 μM

2434 nmoles/g. Values of the accumulation of xanthine and uric acid over the initial 50 min of incubation were used in calculating k_{1HX}, since there was no further xanthine formation after this period; k_{1HX} was 0.0007 ± 0.0002 min^{-1} and the rate constant k_{1H} was therefore 0.0537 ± 0.0048 min^{-1}.

The rate of formation of IMP is given by $dIMP/dt = k_{1H}H - (k_{2HA} + k_{2HG})IMP$. The steady state condition $dIMP/dt = 0$, approximates the maximum concentration of IMP. Inosinate reached a maximum concentration (IMP^{mIMP}) of 24.6 nmoles/g after 5 min incubation (Fig. 9), at which time the concentration of hypoxanthine (H^{mIMP}) was 1940 nmoles/g (Fig. 8). The ratio, k_{2HA}/k_{2HG}, as defined by equation [11], was 5.29 ± 0.21 and substitution into equations [11] and [12] gave k_{2HA} = 3.56 ± 0.46 min^{-1} and k_{2HG} = 0.67 ± 0.08 min^{-1}.

$$[11] \quad \frac{k_{2HA}}{k_{2HG}} = \left[\frac{AMP+ADP+ATP+NAD+\text{Nucleic acid adenine}+AR+A}{GMP+GDP+GTP+\text{Nucleic acid guanine}+G+GR}\right]^{t}$$

$$[12] \quad k_{2HA} + k_{2HG} = k_{1H}\frac{H^{mIMP}}{IMP^{mIMP}}$$

The intermediates in the conversion of IMP to GMP and AMP have been omitted for the simplification of the kinetic analysis. Other experiments (Crabtree and

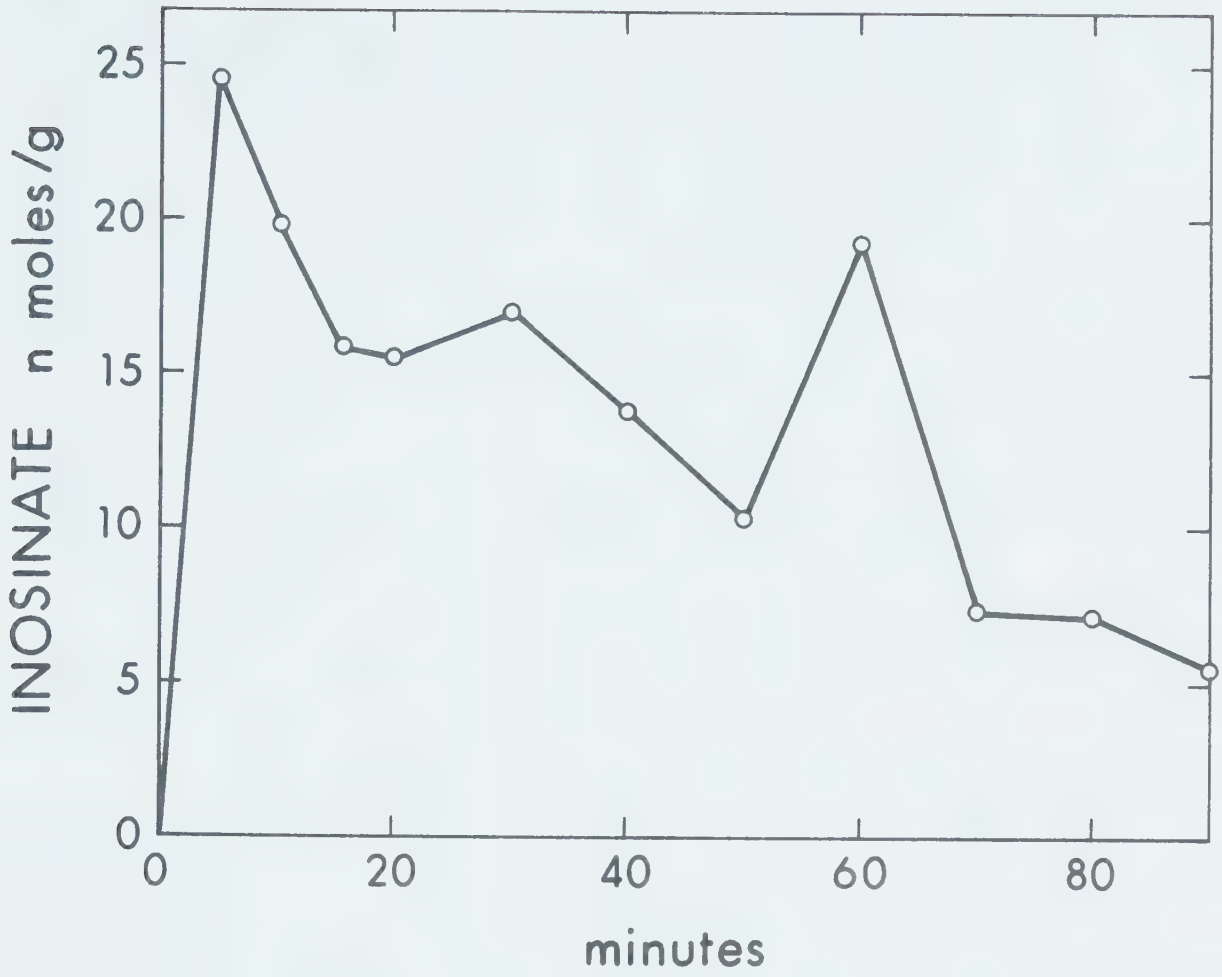


Figure 9. Concentration of radioactive IMP.

Precursor: hypoxanthine-^{14}C, 50 µM initial concentration.

Henderson, '71a) have indicated that adenylosuccinate does not accumulate under the conditions of these experiments. Xanthylate concentrations were routinely determined and found always to be lower than IMP concentration; the maximum concentration of XMP was 15 nmoles/g. Thus the conversion of IMP to AMP represents the sum of reactions catalyzed by adenylosuccinate synthetase (EC 6.3.4.4) and adenylosuccinate lyase (EC 4.3.2.2); and the conversion of IMP to GMP represents the sum of reactions catalyzed by IMP dehydrogenase (EC 1.2.1.14) and GMP synthetase (EC 6.3.5.2). Apparently the first enzymatic reaction of each branch point is rate limiting.

Rate constants for the nucleoside mono- and diphosphate kinase reactions have already been determined using adenine and guanine as radioactive precursors. The steady state concentrations of AMP and ADP were similar for either hypoxanthine or adenine as precursor and the steady state concentrations of GMP and GDP were nearly equivalent for either hypoxanthine or guanine as precursor. By substituting the appropriate constants from Tables 2, 3, and 4 and the initial concentration of hypoxanthine, H^{o} = 2434 nmoles/g, into the symmetrical equations [13] and [14], the rate of formation of ATP plus nucleic acid adenine, and of GTP plus nucleic acid guanine was predicted. The product accumulation predicted by the model

Table 4. Rate constants of hypoxanthine metabolism

Reaction	Rate Constants (min^{-1})
H ⟶ IMP	$k_{1H} = 0.0537 \pm 0.0048$
H ⟶ X	$k_{1HX} = 0.0007 \pm 0.0002$
IMP ⟶ AMP	$k_{2HA} = 3.56 \pm 0.46$
IMP ⟶ GMP	$k_{2HG} = 0.67 \pm 0.08$

EQUATION [13]

$$ATP = H^{\circ}\left[\frac{k_{1H}k_{2HA}}{(k_{1H}+k_{1HX})(k_{2HA}+k_{2HG})} \right.$$

$$- \frac{k_{1H}k_{2HA}k_{2A}k_{3A}}{(k_{1H}+k_{1HX})(k_{2HA}+k_{2HG}-k_{1H}-k_{1HX})(k_{2A}-k_{1H}-k_{1HX})(k_{3A}-k_{1H}-k_{1HX})} e^{-(k_{1H}+k_{1HX})t}$$

$$- \frac{k_{1H}k_{2HA}k_{2A}k_{3A}}{(k_{2HA}+k_{2HG})(k_{1H}+k_{1HX}-k_{2HA}-k_{2HG})(k_{2A}-k_{2HA}-k_{2HG})(k_{3A}-k_{2HA}-k_{2HG})} e^{-(k_{2HA}+k_{2HG})t}$$

$$- \frac{k_{1H}k_{2HA}k_{3A}}{(k_{1H}+k_{1HX}-k_{2A})(k_{2HA}+k_{2HG}-k_{2A})(k_{3A}-k_{2A})} e^{-(k_{2A})t}$$

$$\left. - \frac{k_{1H}k_{2HA}k_{2A}}{(k_{1H}+k_{1HX}-k_{3A})(k_{2HA}+k_{2HG}-k_{3A})(k_{2A}-k_{3A})} e^{-(k_{3A})t} \right]$$

EQUATION [14]

$$GTP = H^{o}\left[\frac{k_{1H}k_{2HG}}{(k_{1H}+k_{1HX})(k_{2HA}+k_{2HG})}\right.$$

$$-\frac{k_{1H}k_{2HG}k_{2G}k_{3G}}{(k_{1H}+k_{1HX})(k_{2HA}+k_{2HG}-k_{1H}-k_{1HX})(k_{2G}-k_{1H}-k_{1HX})(k_{3G}-k_{1H}-k_{1HX})}\ e^{-(k_{1H}+k_{1HX})t}$$

$$-\frac{k_{1H}k_{2HG}k_{2G}k_{3G}}{(k_{2HA}+k_{2HG})(k_{1H}+k_{1HX}-k_{2HA}-k_{2HG})(k_{2G}-k_{2HA}-k_{2HG})(k_{3G}-k_{2HA}-k_{2HG})}\ e^{-(k_{2HG}+k_{2HA})t}$$

$$-\frac{k_{1H}k_{2HG}k_{3G}}{(k_{1H}+k_{1HX}-k_{2G})(k_{2HA}+k_{2HG}-k_{2G})(k_{3G}-k_{2G})}\ e^{-(k_{2G})t}$$

$$\left.-\frac{k_{1H}k_{2HG}k_{2G}}{(k_{1H}+k_{1HX}-k_{3G})(k_{2HA}+k_{2HG}-k_{3G})(k_{2G}-k_{3G})}\ e^{-(k_{3G})t}\right]$$

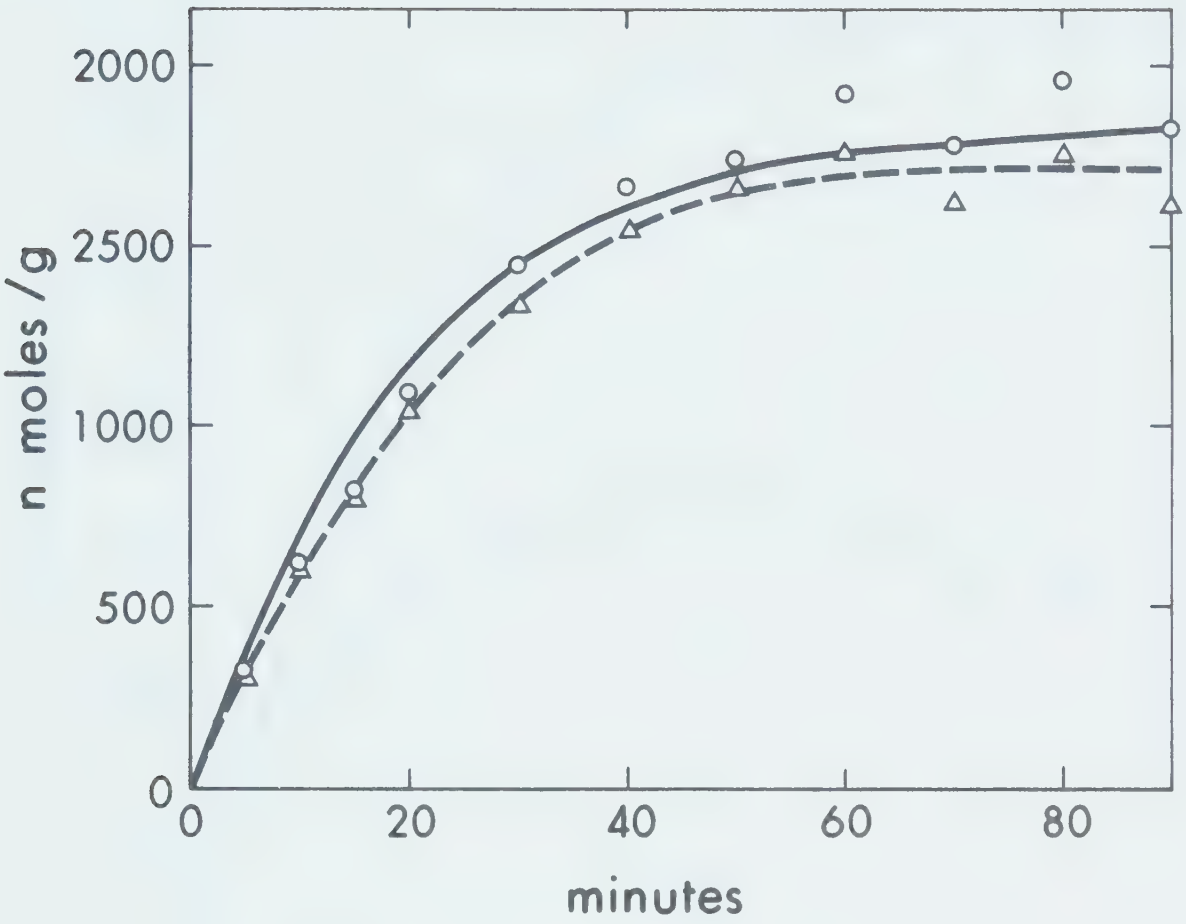


Figure 10. Concentration of radioactive ATP and of ATP plus nucleic acid adenine

Precursor: hypoxanthine-^{14}C, 50 μM initial concentration.
Solid curve was calculated from rate constants in Tables 1 and 4.
(Δ) ATP (dashed curve)
(o) ATP plus nucleic acid adenine

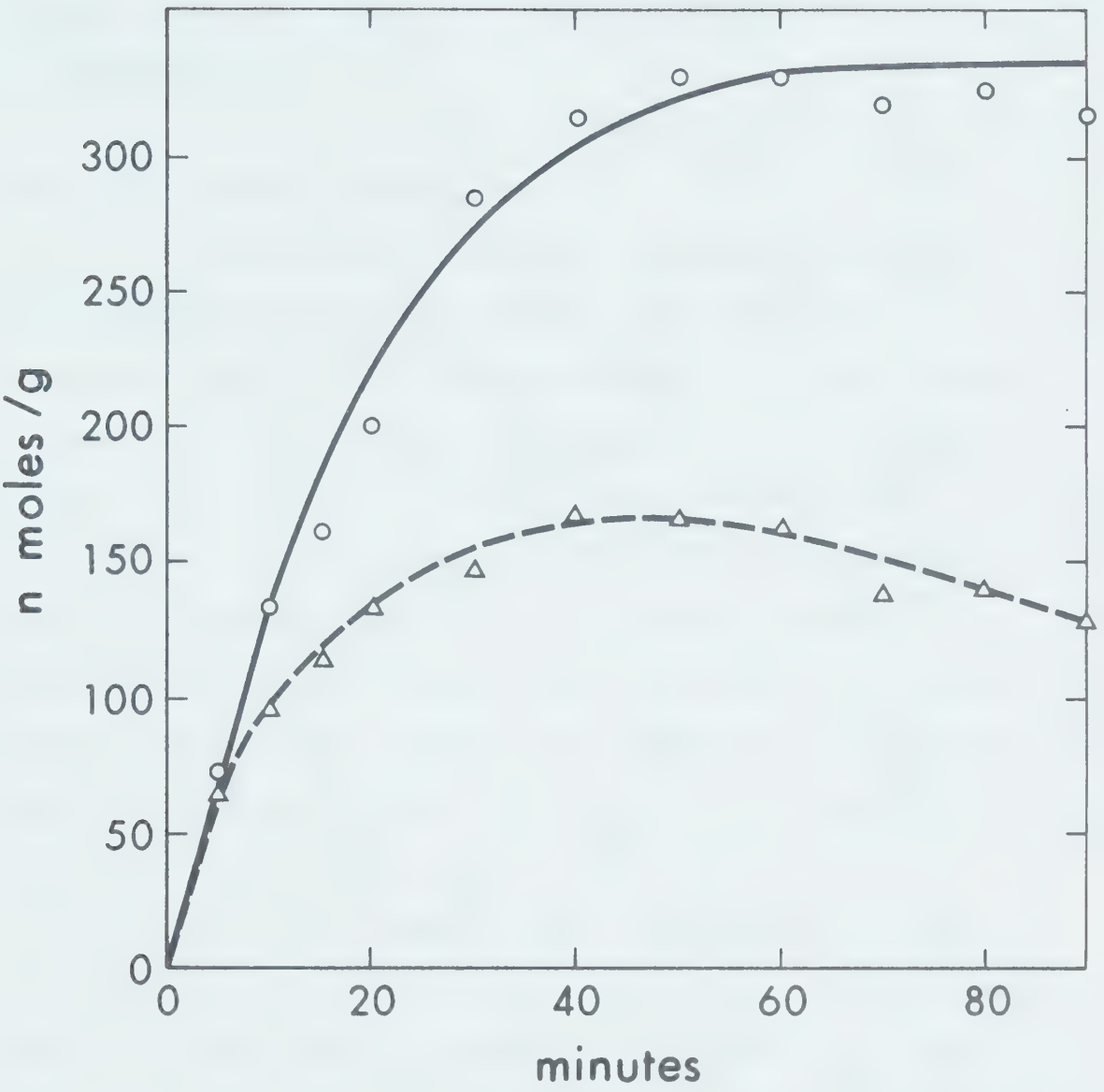


Figure 11. Concentration of radioactive GTP and of GTP plus nucleic acid guanine

Precursor: hypoxanthine-^{14}C, 50 μM initial concentration.
Solic curve was calculated from rate constants in Tables 3 and 4.
(Δ) GTP (dashed curve)
(o) GTP plus nucleic acid guanine.

together with experimentally measured values are given in Figures 10 and 11.

Total acid-soluble nucleotides

The total acid soluble nucleotide profile of cells incubated with 50 μM adenine over a 60 min time course was examined to determine whether the measurements of radioactive nucleotides considered above were representative of the total nucleotide concentrations within the cell. Total acid-soluble and radioactive adenine nucleotides are compared in Table 5. Although nucleoside monophosphate concentrations were too small to be quantitatively measured by liquid chromatography of cell extracts, the concentrations of AMP, IMP and GMP were less than 100 nmoles/g in these experiments. The concentration of radioactive AMP also remained low, attaining its steady state concentration after 5 min incubation. The concentration of ADP (Table 5) rose to a maximum of 533 nmoles/g in 15 min, then dropped to a constant value of approximately 420 nmoles/g between 30 and 60 min incubation. The concentration of radioactive ADP also followed this pattern, attaining a maximum level in 15 min, and remaining essentially constant up to 90 min of incubation (Table 5 and Fig. 3). The close correlation between total and radioactive ADP concentrations may be taken as indirect evidence

Table 5. Comparison of total chemical and radioactive acid-soluble adenosine nucleotide concentrations*

Incubation Time (min)	Total Nucleotides		Radioactive Nucleotides			Net ATP Increase
	ATP	ADP	ATP	ADP	AMP	
	(nmoles/g)					
0	3485	286	0	0	0	0
5	4055	329	559	25.0	7.0	570
15	5725	533	1245	77.6	8.9	2240
30	6615	412	1796	72.2	6.1	3130
60	5825	423	2204	81.8	10.4	2340

* Cells were incubated with 50 μM adenine-^{14}C and concentrations of total nucleotides and radioactive nucleotides were measured at various intervals.

that radioactive and non-radioactive pools are mixed and that the radioactive nucleotides are representative of the total nucleoside mono- and diphosphate concentrations. It is also clear from the concentration of ATP reported in Table 5, that there is de novo synthesis of adenine nucleotides during the course of the incubation, since the radioactive ATP concentration does not account for the increase in total ATP, particularly at 15 and 30 min.

Saturating precursor concentration

Nucleotide synthesis from a saturating concentration (1.0 mM) of adenine and hypoxanthine was also studied in Ehrlich ascites tumor cells. During the course of these experiments the concentration of the purine base precursor remains essentially constant.

Nucleotide synthesis from 1.0 mM adenine as shown in Figure 12, was linear from 30 to 90 min and the rate of nucleotide synthesis during this period was 49.2 ± 4.2 nmoles/g per min. Nucleotide synthesis from 1.0 mM hypoxanthine, as shown in Figure 13, was also linear from 30 to 90 min and the rate of nucleotide synthesis was 24.2 ± 1.5 nmoles /g per min during this period. The formation of AMP, as represented by the sum of radioactivity in AMP, ADP, ATP, NAD and nucleic acid adenine, is also given in Figure 13. The rate of AMP synthesis paralleled that

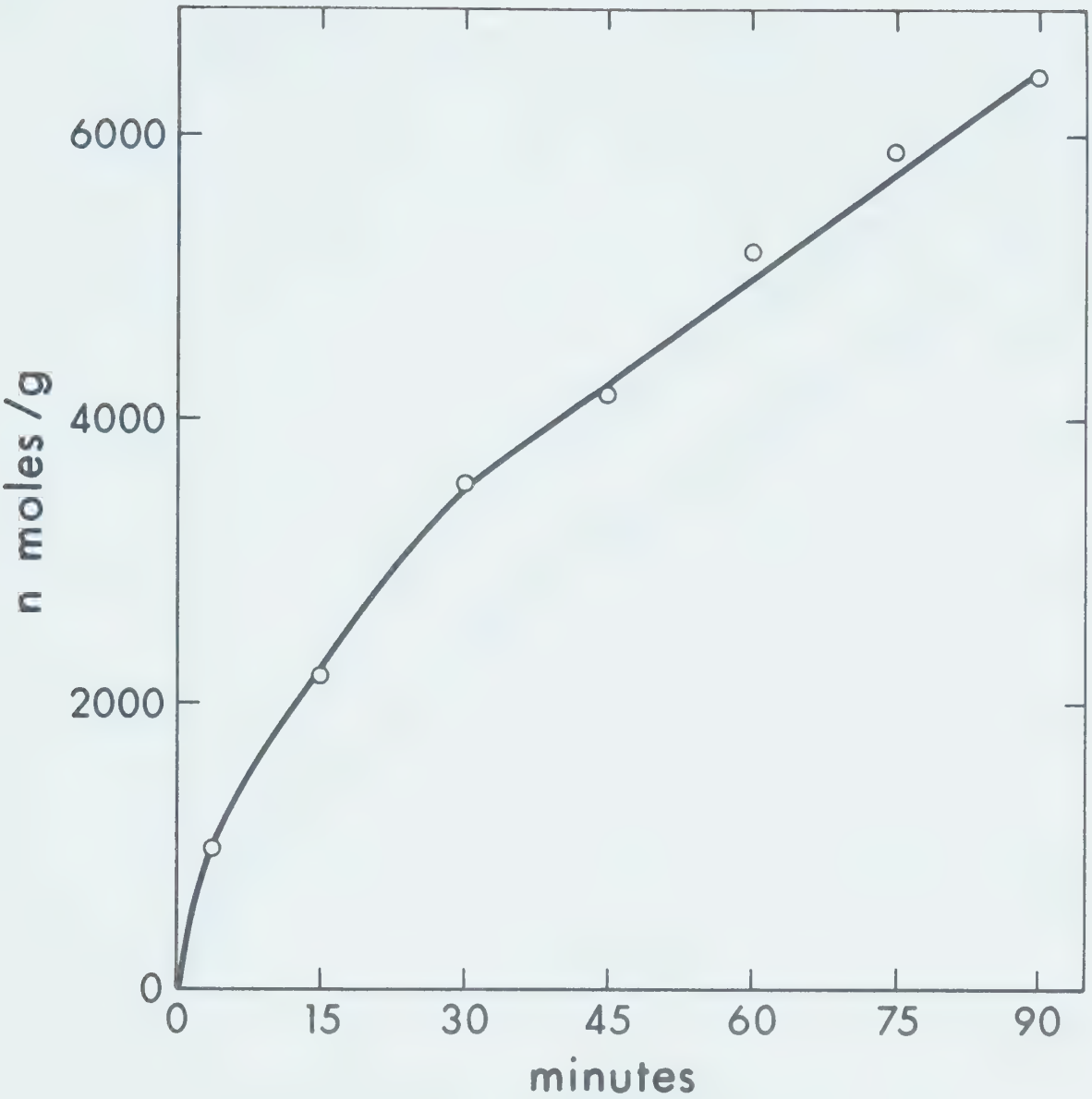


Figure 12. Nucleotide synthesis from 1.0 mM adenine-^{14}C

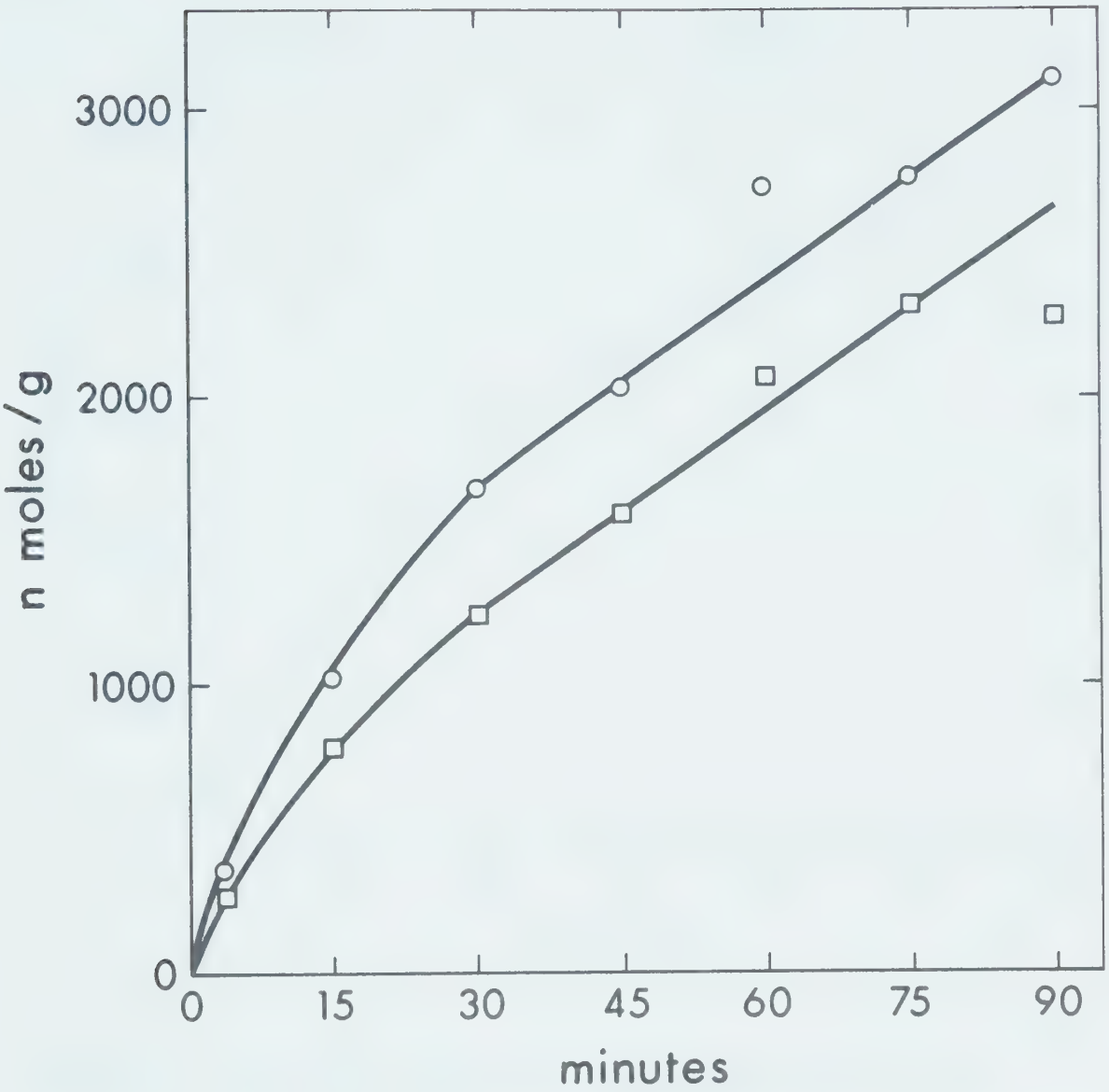


Figure 13. Total nucleotide and adenine nucleotide synthesis from 1.0 mM hypoxanthine-^{14}C

(o) Total nucleotides
(□) Adenine nucleotides

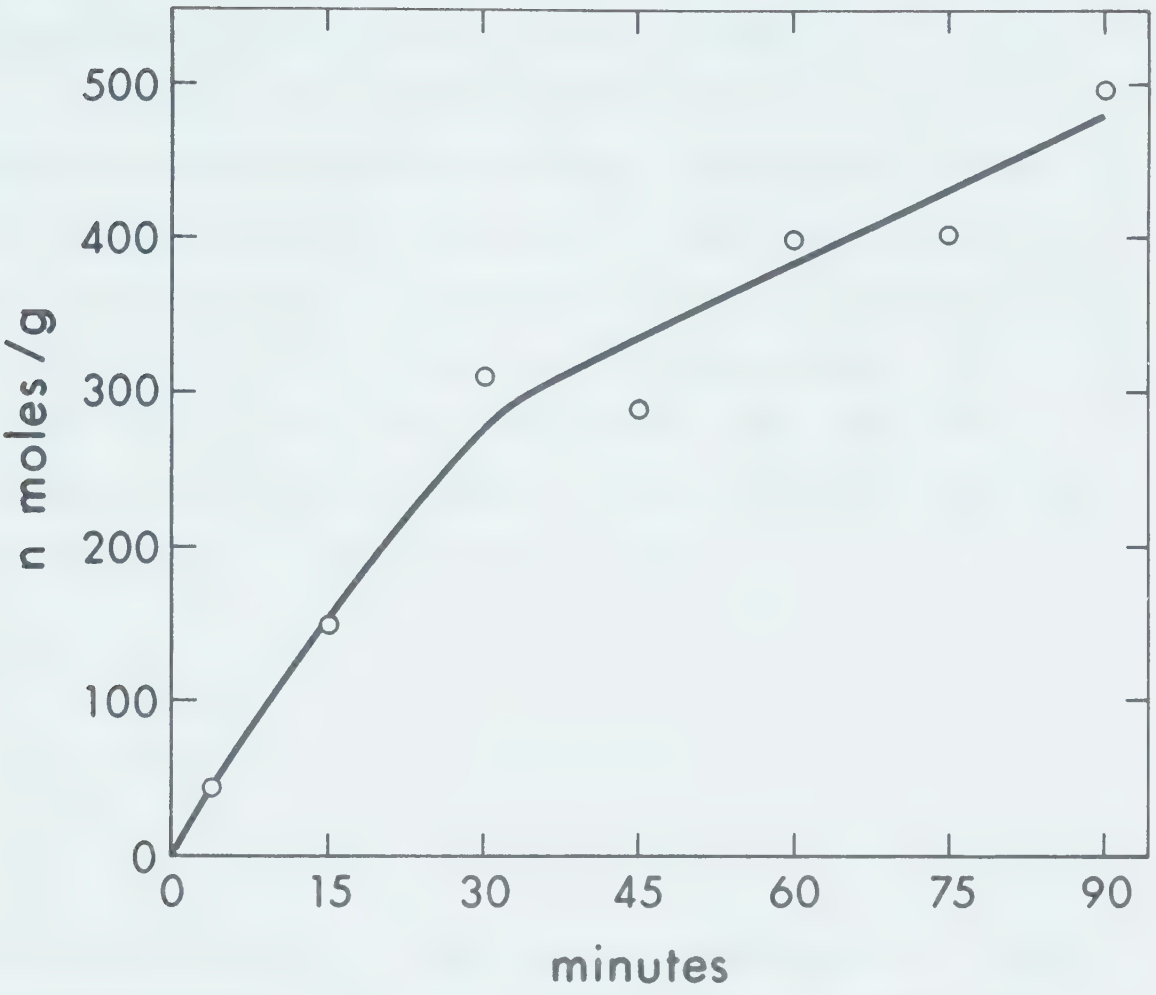


Figure 14. Guanine nucleotide synthesis from 1.0 mM hypoxanthine-^{14}C

of total nucleotide synthesis, having a rate of 24.1 ± 2.7 nmoles/g per min during the linear period from 30 to 90 min.

The rate of GMP synthesis, which is the sum of radioactivity in GMP, GDP, GTP and nucleic acid guanine, is given in Figure 14. The rate of GMP synthesis from 30 to 90 min was 3.2 ± 1.1 nmoles/g per min. The ratio of the rates of AMP and GMP synthesis from hypoxanthine, 24.1/3.2 = 7.5 ± 3.4, is higher than the ratio of the rate constants for these reactions determined at non-saturating substrate concentration, $k_{2A}/k_{2HG} = 5.29 \pm 0.21$.

DISCUSSION

The processes of purine ribonucleotide synthesis and interconversion in intact cells are amenable to kinetic analysis by using a single labelled precursor which may be taken up by the cell and converted to nucleotides. The theoretical rates of nucleoside triphosphate synthesis, predicted by the first order model (Scheme 1), were shown to be an adequate description of the synthesis from labelled precursors of radioactive ATP plus nucleic acid adenine and GTP plus nucleic acid guanine. The model requires that the precursor concentration be less than the saturation level for base uptake by the cell and conversion

to nucleotide product, so that the proportionality between rate of nucleotide synthesis and substrate concentration is maintained. The specific activity of each base precursor was assumed to remain constant. Rate constants for all of the reactions in Scheme 1 have been obtained.

The analysis of reactions having a common substrate, assesses the relative activity of alternate enzymatic reactions in the cells. The formation of IMP from AMP was only a fraction of that converted to ADP and ATP, as represented by the ratio $k_{2A}/k_{2AH} = 68$. Similarly the formation of IMP from GMP was a small fraction of the conversion of GMP to GDP and GTP, $k_{2G}/k_{2GH} = 33$, although in the last 30 min this ratio assumed the value of 16.9 indicating that a greater proportion of GMP was being converted to IMP. Activities of the respective nucleoside monophosphate kinases were thus effectively greater than the activities of adenylate deaminase (EC 3.5.4.6) or guanylate reductase (EC 1.6.6.8).

Comparison of the rate constants for the deamination of AMP, k_{2AH} and conversion of IMP to GMP, k_{2HG}, indicates that for equivalent substrate concentrations, the former reaction would be rate limiting. Also the rate constants for GMP conversion to IMP, k_{2GH}, and conversion of IMP to AMP, k_{2HA}, suggest that the conversion of GMP to adenine nucleotides is limited by GMP reductase (EC 1.6.6.8)

activity. These results are in agreement with the semi-quantitative findings of Crabtree and Henderson ('71a).

An estimation of the relative use of hypoxanthine and guanine as precursors of xanthine and uric acid may be obtained by comparing the rate constants for hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8), k_{1H} and k_{1G}, with those for xanthine oxidase (EC 1.2.3.2), k_{1HX}, and for guanine deaminase (EC 3.5.4.3), k_{1GX}. The fraction of hypoxanthine which may be oxidized to xanthine relative to its conversion to IMP is given by: $k_{1HX}/(k_{1H} + k_{1HX}) = 0.013$. Similarly the fraction of guanine which may be deaminated to xanthine, also relative to its conversion to nucleotides, is given by: $k_{1GX}/(k_{1G} + k_{1GX}) = 0.157$. Comparison of these ratios indicates that at equal rates of hypoxanthine and guanine formation in the cell, guanine would be a better precursor of xanthine and uric acid than hypoxanthine by a factor of 12.

Interesting differences have been observed between the rates of nucleotide synthesis at 50 μM purine base concentration calculated from first order rate constants and the rates of nucleotide synthesis at saturating base concentrations. The rate of nucleotide synthesis from 50 μM adenine is given by $-dA/dt = k_{1A}A$, or 149 nmoles /g per min, whereas the steady state rate of nucleotide synthesis from 1.0 mM adenine was only 49.2 nmoles/g per min.

Similarly, the rate of nucleotide synthesis from 50 μM hypoxanthine (given by $k_{1H}H^{\circ}$), was 134 nmoles/g per min, whereas the steady state rate of nucleotide synthesis from 1.0 mM hypoxanthine was 24.2 nmoles/g per min. These results suggest that in Ehrlich ascites tumor cells, after the first 30 min of incubation, nucleotide synthesis from saturating concentrations of purine bases was limited by factors not expressed at non-saturating precursor concentrations. Furthermore, at saturating precursor concentrations, the rate of AMP synthesis from hypoxanthine, 24.1 nmoles/g per min, was limited by the rate of conversion of hypoxanthine to IMP, 24.2 nmoles/g per min. Since the rate of AMP synthesis from IMP was almost the same as the rate of IMP synthesis from hypoxanthine, the rate of GMP synthesis from IMP is relatively independent of the rate of AMP formation from IMP.

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CHAPTER 3

INHIBITION OF PURINE METABOLISM — COMPUTER-ASSISTED ANALYSIS OF DRUG EFFECTS

INTRODUCTION

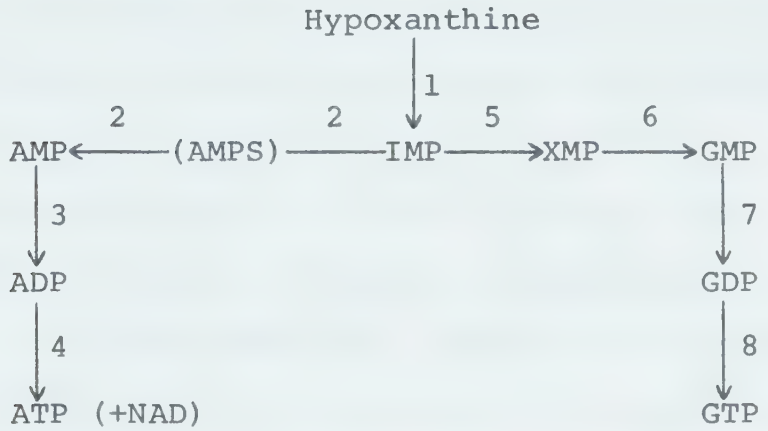
Purine ribonucleotides are essential for cell growth and function, and the pathways of their synthesis and conversion have proven to be fruitful areas of chemotherapeutic attack. Although the effects of drugs on pathways or groups of enzymes of purine metabolism can be measured relatively easily in intact cells *in vitro* or *in vivo*, the determination of effects of drugs on individual enzymes in such complex systems is much more difficult. We report here an analytical procedure by which the effects of drugs on the apparent rates of several individual enzymes of purine metabolism may be determined. The analysis has been facilitated by the use of a suitable computer program written in the APL/360 language.

METHODS AND RESULTS

Experimental system

In order to investigate the effects of drugs on a wide range of enzymes of purine ribonucleotide synthesis and interconversion, hypoxanthine-8-^{14}C (52.0 mCi/mmole, Schwarz BioResearch) was employed as precursor and its

metabolic transformations were examined. The routes of hypoxanthine metabolism which were studied are shown in Scheme I.



Scheme I. Routes of hypoxanthine metabolism.

Ehrlich ascites tumor cells were collected and diluted in Fischer's medium modified to contain 25 mM phosphate buffer, pH 7.4, without bicarbonate, to make a 2.5 per cent cell suspension by volume. The tumor cell suspension (80 µl), additional medium (5 µl) and test compounds (10 µl, 1 mM final concentration) were incubated in small plastic tubes (10 x 75 mm, Falcon Plastics) for 20 min at 37° with shaking. Hypoxanthine-^{14}C was then added (5 µl, 0.1 mM final concentration) and the incubation continued. After 60 min, 5 µl of 4.2 M cold perchloric acid was added to each tube and the cell extract was neutralized by the

addition of 5 µl of 4.42 M KOH. Samples were chilled, centrifuged and 10 µl of the supernatant was spotted for chromatography.

Purine ribonucleotides were separated by thin-layer chromatography on polyethyleneimine-cellulose according to the method of Crabtree and Henderson (1). The amount of radioactivity in the following purine nucleotides was measured: GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP and NAD. Results of duplicate measurements were averaged.

Adenylosuccinate (AMPS) was not isolated and hence the adenylosuccinate synthetase and adenylosuccinate lyase reactions are not distinguished. Previous studies (1) have suggested that the former is rate limiting for the conversion of IMP to AMP.

Ehrlich ascites tumor cells were incubated for 60 min in modified Fischer's medium with 100 µM hypoxanthine-^{14}C, in the presence and absence of drugs. Under these conditions 82.6 per cent of the radioactivity was converted to nucleotides, 1.6 per cent was oxidized directly to xanthine plus uric acid, 0.8 per cent was converted via nucleotides to other purine bases and ribonucleosides, 11.8 per cent was converted to acid-insoluble material and 3.2 per cent remained as hypoxanthine.

Both because the screening efforts have been directed primarily to reactions of ATP and GTP synthesis from

hypoxanthine and because the amounts of radioactivity in bases and nucleosides were so small, the latter have been ignored in this analysis. As a simplification, the radioactivity in the acid-insoluble fraction (nucleic acids) derived from ATP and GTP has also not been included in this analysis.

Enzyme rates

The apparent rate of each of the enzymic reactions illustrated in Scheme I is taken to be represented by the sum of the radioactivity in all ribonucleotides which occur further along the pathway. Thus reactions 1-8 in Scheme I are represented by the arithmetic sums which are shown in Table I. The enzyme names employed in Table I correspond to the metabolic processes measured and are not necessarily intended to imply the involvement of only one enzyme. For example "ADP kinase" represents total ATP formation from ADP.

In addition, three other parameters of purine ribonucleotide metabolism were computed. The ratios of radioactive nucleoside triphosphate (ATP and GTP) to their respective mono- and diphosphates gave a measure of what may be called the "energy status" of the radioactive adenine and guanine nucleotide pools. The relative rates of conversion of IMP to adenine and guanine nucleotides were also calculated. These functions are shown in Table 2.

Table 1. Summations representing apparent rates of enzymatic processes in Scheme I

Process	Enzyme system	Summation
1	Hypoxanthine phospho-ribosyltransferase	ATP+ADP+AMP+NAD+ GTP+GDP+GMP+XMP+IMP
2	AMPS synthetase plus lyase	ATP+ADP+AMP+NAD
3	AMP kinase	ATP+ADP+NAD
4	ADP kinase	ATP+NAD
5	IMP dehydrogenase	GTP+GDP+GMP+XMP
6	GMP synthetase	GTP+GDP+GMP
7	GMP kinase	GTP+GDP
8	GDP kinase	GTP

Table 2. Additional parameters of purine ribonucleotide metabolism

Parameter	Function
Adenine nucleotide energy status	ATP ÷ (AMP+ADP)
Guanine nucleotide energy status	GTP ÷ (GMP+GDP)
Adenine/Guanine nucleotides	(ATP+ADP+AMP+NAD) ÷ (XMP+GMP+GDP+GTP)

Computation of enzyme rates

While it is possible to compute the apparent rates of the various enzymes illustrated in Scheme I by use of manual techniques, the advantages of accuracy and speed which accompany a computer-assisted analysis suggested that the latter approach would be more valuable. The program was written in APL/360, a terminal-oriented language devised by Iverson (2); the computer used was an IBM 360/67. The program first requests input of an identification number by which the drug being tested may be recorded on the print-out; control data are identified by the numeral 1. The values of the radioactivity in GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP and NAD are then entered for control and drug-treated cells. Any number of different drugs may be tested against a single set of control values in a single run. Any units may be employed for the quantities of the different nucleotides (counts per minute, micromoles per gram of cells, etc.) provided that the method of expressing the results is consistent within any given run.

As an example of the use of these procedures, Table 3 gives the apparent rates of enzymes of hypoxanthine metabolism in cells incubated in the presence and absence of mycophenolic acid, a known inhibitor of IMP dehydrogenase (3).

Table 3. The apparent rates of enzymes and parameters of purine metabolism [a].

Parameter	Control (counts/min)	Mycophenolic acid (counts/min)
Hypoxanthine phospho-ribosyltransferase	11473	12174
AMPS synthetase plus lyase	9271	10507
AMP kinase	8867	10242
ADP kinase	7998	9265
IMP dehydrogenase	1055	137
GMP synthetase	972	108
GMP kinase	835	86
GDP kinase	702	38
Adenine nucleotide energy status	6.027	7.387
Guanine nucleotide energy status	2.600	0.543
Adenine/Guanine nucleotides	8.788	76.693

[a]Cells were incubated with 100 μM hypoxanthine-^{14}C for 60 minutes in the presence and absence of 1.0 mM mycophenolic acid.

Computation of fractional inhibition

Fractional inhibition of each of the enzymes studied may be obtained by division of the apparent rates in the presence of the drugs by the corresponding control values. This process is also performed by the program and Table 4 shows the results of such calculations applied to the data of Table 3.

Inspection of these data shows that the primary effect of mycophenolic acid was inhibition of IMP dehydrogenase, the rate of which was approximately 10 per cent that of hypoxanthine phosphoribosyltransferase, the preceding enzyme in the pathway (Scheme I). The enzymes synthesizing ATP from IMP have increased rates, which may be due to an increased availability of IMP for AMPS synthetase and lyase due to inhibition of the alternative pathway of IMP metabolism. The increased adenine/guanine nucleotide ratio also reflects inhibition of IMP dehydrogenase. These data also suggest that the conversion of GDP to GTP is significantly inhibited by mycophenolic acid, since the fractional rate of this process is about half that of the previous step.

Computation of per cent inhibition

The analysis applied above to the data of Table 4 remains only semiquantitative because the apparent fractional

Table 4. Fractional inhibition by mycophenolic acid

Parameter	Mycophenolic acid (Fraction of control)
Hypoxanthine phosphoribosyl-transferase	1.061
AMPS synthetase plus lyase	1.133
AMP kinase	1.155
ADP kinase	1.158
IMP dehydrogenase	0.130
GMP synthetase	0.111
GMP kinase	0.103
GDP kinase	0.054
Adenine nucleotide energy status	1.226
Guanine nucleotide energy status	0.209
Adenine/Guanine nucleotides	8.727

inhibition of any particular enzyme (except hypoxanthine phosphoribosyltransferase) is influenced by the rate of the enzyme(s) preceding it in Scheme I. Thus 90 per cent inhibition of IMP dehydrogenase leads to an apparent 90 per cent inhibition of GMP synthetase, GMP kinase and GDP kinase simply because there is little radioactivity in the guanine nucleotides due to the block in IMP dehydrogenase. This drawback is corrected in the next step of the program, which calculates the per cent inhibition of each individual process and compensates for inhibition of preceding steps in the pathways of metabolism.

Per cent inhibition is given by:

$$P = \left[\frac{f^p - f}{f^p} \right] \times 100 = (1 - f/f^p) \times 100$$

where P is the per cent inhibition, f is the fractional inhibition of the process considered and f^p is the fractional inhibition of the preceding process. The value of f^p for conversion of hypoxanthine to IMP is 1. The sequence of processes illustrated in Fig. I defines the choice of other f^p values. The results of calculating per cent inhibition for the different enzymic processes are shown in Table 5. These data are obtained from Table 4 and represent inhibition by mycophenolic acid. Negative values

Table 5. Percent inhibition by mycophenolic acid

Parameter	Computation	Percent inhibition
Hypoxanthine phosphoribosyltransferase	$\left[1-\frac{1.061}{1}\right] \times 100$	-6.1
AMP synthetase plus lyase	$\left[1-\frac{1.133}{1.061}\right] \times 100$	-6.8
AMP kinase	$\left[1-\frac{1.155}{1.133}\right] \times 100$	-1.9
ADP kinase	$\left[1-\frac{1.158}{1.155}\right] \times 100$	-0.3
IMP dehydrogenase	$\left[1-\frac{0.130}{1.061}\right] \times 100$	87.7
GMP synthetase	$\left[1-\frac{0.111}{0.130}\right] \times 100$	14.6
GMP kinase	$\left[1-\frac{0.103}{0.111}\right] \times 100$	7.2
GDP kinase	$\left[1-\frac{0.054}{0.103}\right] \times 100$	47.6

for percentage inhibition represent augmented processes.

It may be seen from Table 5 that the suggestions based on careful scrutiny in Table 4 are confirmed. IMP dehydrogenase is greatly inhibited by mycophenolic acid and there is, in addition, marked inhibition of GDP kinase.

DISCUSSION

Studies of effects of drugs on purine metabolism in intact cells and tissues have gradually come to involve the measurement of radioactivity in more and more ribonucleotides, and Hill *et al.* (4,5), for example, have recently done such measurements on from six to eight compounds. In most cases, however, it still has been found difficult to assign a drug effect to a specific enzyme on the basis of such results and even more difficult to determine its effect quantitatively on a particular enzyme in the intact cell.

The methods presented here for computation of apparent rates of enzymes in intact cells and of drug effects, expressed in per cent inhibition of individual enzymes, appear to have overcome many of these difficulties. This procedure is sensitive to small changes in apparent rates of the enzymes involved, to weak effects of drugs

and the use of a terminal-operated computer for analysis of the data lends the added advantages of speed and convenience. The data presented here for mycophenolic acid confirm previous reports (3) that it inhibits IMP dehydrogenase, but in addition it has also been shown to inhibit GDP kinase. This procedure has also been applied to 92 purine analogues and derivatives (6).

The relationship between the apparent enzyme rates, measured here on the basis of total radioactivity measurements and the true rates depends on such considerations as mixing of radioactive and nonradioactive ribonucleotides and relative sizes of nucleotide pools and may vary for different reactions and in different cells. However, other studies with Ehrlich ascites tumor cells (reference 1 and unpublished data) suggest that radioisotope flow from IMP to ATP and GTP is virtually equivalent to the observed changes in chemical composition of the triphosphate pools.

Extension or modification of the experimental and computational methods could broaden the applicability of this approach. The consideration of radioactivity incorporated into the nucleic acid fraction, derived from ATP and GTP, has proved valuable in some situations where the contribution of this fraction cannot be ignored. The analysis of drug effects <u>in vivo</u> is an important extension

of the study of drug effects *in vitro* and the methodology permitting such an analysis is being developed. Modification of the details of computation would also render this method applicable to screening programs using labeled purine nucleotide precursors other than hypoxanthine.

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CHAPTER 4

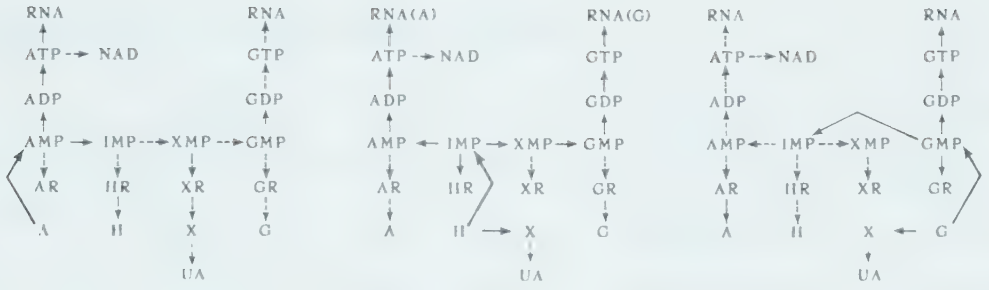
COMPUTER ANALYSIS OF PATHWAYS OF PURINE METABOLISM

INTRODUCTION

An analytical procedure was presented in Chapter 3 by which effects of drugs on apparent activities of several individual enzymes of hypoxanthine-^{14}C metabolism can be determined. This basic procedure is here expanded to include additional enzymes of hypoxanthine-^{14}C metabolism, a wide range of enzymes of adenine-^{14}C and guanine-^{14}C metabolism, and the reactions of ATP-^{14}C catabolism. The calculations of apparent enzyme activities are based on measurements of radioactivity in purine bases, ribonucleosides, ribonucleotides and nucleic acids; the computer programs which perform these calculations are written in APL/360, and are given here.

METHOD OF ANALYSIS

Unidirectional pathways of adenine-^{14}C, hypoxanthine-^{14}C, and guanine-^{14}C metabolism are shown in Scheme 1.



Adenine metabolism Hypoxanthine metabolism Guanine metabolism

SCHEME 1

Methods for the measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides have been described (1), as have methods for measurement of radioactivity in nucleic acid adenine and nucleic acid guanine (Chapter 2). The apparent activities of each enzymatic reaction in Scheme 1 are represented by the arithmetic sums shown in Tables 1A, 2A, and 3A, for each precursor.

In addition, other parameters of purine ribonucleotide metabolism were also computed, in particular the relative activities of processes having a common substrate, such as the ratio, AMP kinase/AMP deaminase. These additional parameters are shown in Tables 1B, 2B and 3B.

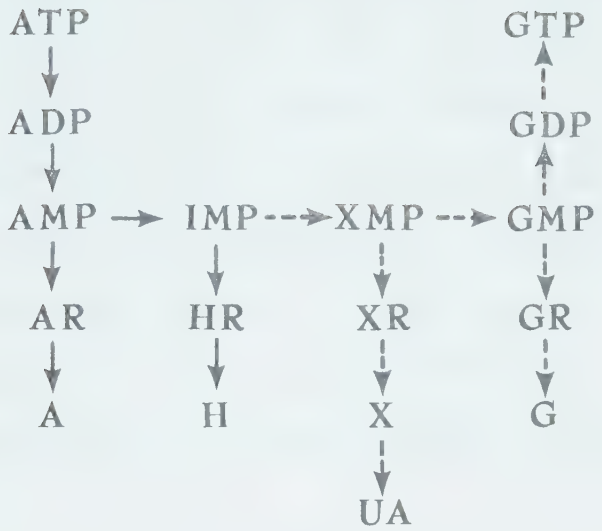
The calculation of apparent activities and additional parameters of the enzymatic processes of Tables 1, 2, and 3, are computed by the programs AMETAB, HMETAB and GMETAB, which compute parameters for adenine, hypoxanthine, and guanine metabolism, respectively, and which are written in APL/360. The format for data entry into programs is

clearly specified in each program.

In order to measure drug effects, or other variations in incubation conditions, apparent enzyme activities, fractional inhibition, and percent inhibition of individual reactions of purine metabolism are calculated by the method presented in Chapter 3. When hypoxanthine is used as precursor, the program HSCREEN performs the computations outlined in Chapter 3 and program HSCREENNA is a modification which includes in the analysis radioactivity measured in nucleic acid adenine and nucleic acid guanine. When adenine is used as precursor, the program ASCREEN, which is an extension of AMETAB, computes these parameters from radioactivity measured in nucleic acid adenine, ribonucleotides, ribonucleosides and bases.

Catabolism of ATP

The catabolism of purine ribonucleotides has been investigated in this laboratory, and in collaboration with C. A. Lomax a computer analysis was developed which calculates the enzymatic activities of the reactions of ATP catabolism shown in Scheme 2.



ATP catabolism

SCHEME 2

In a typical experiment, cells containing radioactive ATP were obtained by incubating them with adenine-^{14}C. Cells were then washed and resuspended in a fresh medium which induced nucleotide breakdown (for example, catabolism can be induced by 2-deoxyglucose), and radioactivity in all the compounds of Scheme 2 measured over a time course. Coformycin was present in the catabolic phase of these experiments at concentrations that completely inhibited adenosine deaminase (2).

The apparent enzymatic activities of ATP catabolism are obtained from the arithmetic sums of radioactivity in products given in Table 4A. At the start of the catabolic phase, radioactivity is present not only in ATP, but also other metabolites; thus the zero time apparent activities are subtracted from all subsequent activities. The ratio of activities having common substrates are calculated as shown in Table 4B. The program ATPCATAB, also written in APL/360, conveniently performs these calculations.

DISCUSSION

In Schemes 1 and 2 the solid arrows denote reactions through which pass significant amounts (roughly, more than 5% of that converted to nucleotides) of radioactivity derived from each of the precursors indicated. Reactions through which smaller amounts of radioactivity pass are indicated by dotted lines. For example, AMP deaminase activity may be measured using adenine as precursor, although the ratio of AMP deaminase to AMP kinase was 1/68 in Ehrlich ascites tumor cells (Chapter 2), and little radioactivity was found in IMP and other compounds which were products of AMP deaminase. Enzymatic reactions involving IMP, however, may be measured using hypoxanthine as

precursor, for example, IMP dehydrogenase and AMPS synthetase plus lyase; likewise, guanine may be used to measure GMP reductase. Thus by using one or more radioactive precursors a quantitative measure of the activities of virtually all of the individual enzymes of purine metabolism in intact cells may be obtained.

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Table 1A. Summations representing apparent activities of enzymatic processes of adenine metabolism.

Enzyme System	Summation
Adenine phosphoribosyl-transferase	AMP+ADP+ATP+NAD+RNA+IMP+XMP+GMP+GDP+GTP+AR+H+HR+X+XR+G+GR+UA
AMP kinase	ADP+ATP+NAD+RNA
ADP kinase	ATP+NAD+RNA
RNA polymerase	RNA
AMP deaminase	IMP+XMP+GMP+GDP+GTP+H+HR+X+XR+G+GR+UA
IMP dehydrogenase	XMP+GMP+GDP+GTP+X+XR+G+GR+UA
XMP aminase	GMP+GDP+GTP+G+GR
GMP kinase	GDP+GTP
GDP kinase	GTP
AMP dephosphorylase	AR
IMP dephosphorylase	H+HR
XMP dephosphorylase	X+XR+UA
GMP dephosphorylase	G+GR
HR phosphorylase	H
XR phosphorylase	X+UA
GR phosphorylase	G
Xanthine oxidase	UA

Table 1B. Additional parameters of adenine metabolism.

Parameter	Function
Total 'A' nucleotides	AMP+ADP+ATP+NAD+RNA
A energy status	ATP÷(AMP+ADP)
Total 'G' nucleotides	IMP+XMP+GMP+GDP+GTP
G energy status	GTP÷(GMP+GDP)
A/G nucleotide ratio	(AMP+ADP+ATP+NAD+RNA)÷(GMP+GDP+GTP+XMP+IMP)
AMP kinase/AMP deaminase	(ADP+ATP+NAD+RNA)÷(IMP+XMP+GMP+GDP+GTP+H+HR+X+XR+G+GR+UA)
Total nucleotide catabolism	AR+H+HR+X+XR+UA+G+GR
Fraction nucleotide catabolism	(AR+H+HR+X+XR+UA+G+GR)÷(AMP+ADP+ATP NAD+RNA+IMP+XMP+GMP+GDP+GTP+AR+H+HR+X+XR+UA+G+GR)
Fraction AMP catabolism	AR÷(AMP+ADP+ATP+NAD+RNA+AR)
Fraction IMP catabolism	(HR+H)÷(H+HR+IMP+XMP+GMP+GDP+GTP+X+XR+UA+G+GR)
Fraction XMP catabolism	(X+XR+UA)÷(X+XR+UA+XMP+GMP+GDP+GTP)
Fraction GMP catabolism	(G+GR)÷(G+GR+GMP+GDP+GTP)
Fraction HR catabolism	H÷(H+HR)
Fraction XR catabolism	(X+UA)÷(X+UA+XR)
Fraction GR catabolism	G÷(G+GR)
Fraction ATP incorporation into nucleic acids	RNA÷(RNA+ATP+NAD)

Table 2A. Summations representing apparent activities of enzymatic processes of hypoxanthine metabolism.

Enzyme System	Summation
Hypoxanthine phospho-ribosyltransferase	IMP+AMP+ADP+ATP+NAD+XMP+GMP+GDP+GTP+A+AR+HR+X+XR+G+GR+RNA(A)+RNA(G)
AMPS synthetase + lyase	AMP+ADP+ATP+NAD+RNA(A)+AR+A
AMP kinase	ADP+ATP+NAD+RNA(A)
ADP kinase	ATP+NAD+RNA(A)
IMP dehydrogenase	XMP+GMP+GDP+GTP+XR+G+GR+RNA(G)
XMP aminase	GMP+GDP+GTP+G+GR+RNA(G)
GMP kinase	GDP+GTP+RNA(G)
GDP kinase	GTP+RNA(G)
RNA polymerase	RNA(A)+RNA(G)
AMP dephosphorylase	A+AR
IMP dephosphorylase	HR
XMP dephosphorylase	XR
GMP dephosphorylase	G+GR
AR phosphorylase	A
GR phosphorylase	G
Xanthine oxidase	X+UA

Table 2B. Additional parameters of hypoxanthine metabolism.

Parameter	Function
Total 'A' nucleotides	AMP+ADP+ATP+NAD+RNA(A)
A energy status	ATP÷(AMP+ADP)
Total 'G' nucleotides	XMP+GMP+GDP+GTP+RNA(G)
G energy status	GTP÷(GDP+GMP)
A/G nucleotide ratio	(AMP+ADP+ATP+NAD+RNA(A))÷(XMP+GMP+GDP+GTP+RNA(G))
AMPS synth./IMP dehyd.	(AMP+ADP+ATP+NAD+RNA(A)+A+AR)÷(XMP+GMP+GDP+GTP+XR+G+GR+RNA(G))
HGPRTASE/X oxidase	(IMP+AMP+ADP+ATP+NAD+RNA(A)+XMP+GMP+GDP+GTP+RNA(G)+A+AR+HR+XR+G+GR)÷(X+UA)
RNA/total NTP	(RNA(A)+RNA(G))÷(ATP+NAD+GTP+RNA(A)+RNA(G))
RNA(A)/ATP synthesis	(RNA(A))÷(ATP+NAD+RNA(A))
RNA(G)/GTP synthesis	(RNA(G))÷(GTP+RNA(G))
Total nucleotide catabolism	A+AR+HR+G+GR+XR
Fraction nucleotide catabolism	(A+AR+HR+G+GR+XR)÷(IMP+AMP+ADP+ATP+NAD+RNA(A)+XMP+GMP+GDP+GTP+RNA(G)+A+AR+HR+G+GR+XR)
Fraction AMP catabolism	(A+AR)÷(AMP+ADP+ATP+RNA(A)+A+AR+NAD)
Fraction IMP catabolism	HR÷(IMP+AMP+ADP+ATP+NAD+RNA(A)+XMP+GMP+GDP+GTP+A+AR+HR+G+GR+XR+RNA(G))
Fraction XMP catabolism	XR÷(XMP+GMP+GDP+GTP+G+GR+XR+RNA(G))
Fraction GMP catabolism	(G+GR)÷(GMP+GDP+GTP+G+GR+RNA(G))
Fraction AR catabolism	A÷(A+AR)
Fraction GR catabolism	G÷(G+GR)

Table 3A. Summations representing apparent activities of enzymatic processes of guanine metabolism.

Enzyme System	Summation
Guanine phosphoribosyl-transferase	GMP+GDP+GTP+RNA+IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR+GR
GMP kinase	GDP+GTP+RNA
GDP kinase	GTP+RNA
RNA polymerase	RNA
GMP reductase	IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR
IMP dehydrogenase	XMP+XR
AMPS synthetase + lyase	AMP+ADP+ATP+NAD+A+AR
AMP kinase	ADP+ATP+NAD
ADP kinase	ATP+NAD
AMP dephosphorylase	A+AR
IMP dephosphorylase	H+HR
XMP dephosphorylase	XR
GMP dephosphorylase	GR
AR phosphorylase	A
HR phosphorylase	H
Guanase	X+UA
Xanthine oxidase	UA

Table 3B. Additional parameters of guanine metabolism.

Parameter	Function
Total 'G' nucleotides	GMP+GDP+GTP+RNA
G energy status	GTP÷(GMP+GDP)
Total 'A' nucleotides	IMP+XMP+AMP+ADP+ATP+NAD
A energy status	ATP÷(AMP+ADP)
G/A nucleotide ratio	(GMP+GDP+GTP+RNA)÷(IMP+XMP+AMP+ADP+ATP+NAD)
GMP kinase/GMP reductase	(GDP+GTP+RNA)÷(IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR)
HGPRTASE/guanase	(GMP+GDP+GTP+RNA+IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR+GR)÷(X+UA)
Total nucleotide catabolism	A+AR+H+HR+XR+GR
Fraction nucleotide catabolism	(A+AR+H+HR+XR+GR)÷(GMP+GDP+GTP+RNA+IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR+GR)
Fraction AMP catabolism	(A+AR)÷(A+AR+AMP+ADP+ATP+NAD)
Fraction IMP catabolism	(H+HR)÷(IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR)
Fraction XMP catabolism	XR÷(XMP+XR)
Fraction GMP catabolism	GR÷(GMP+GDP+GTP+RNA+IMP+XMP+AMP+ADP+ATP+NAD+A+AR+H+HR+XR+GR)
Fraction AR catabolism	A÷(A+AR)
Fraction HR catabolism	H÷(H+HR)
Fraction GTP incorporated into nucleic acid	RNA÷(GTP+RNA)

Table 4A. Summations representing apparent activities of enzymatic processes of ATP catabolism.

Enzyme System	Summation
ATP dephosphorylase	ADP+AMP+IMP+XMP+GMP+GDP+GTP+A+AR+H+HR+X+XR+UA+G+GR
ADP dephosphorylase	AMP+IMP+XMP+GMP+GDP+GTP+A+AR+H+HR+X+XR+UA+G+GR
AMP deaminase	IMP+XMP+GMP+GDP+GTP+H+HR+X+XR+UA+G+GR
IMP dehydrogenase	XMP+GMP+GDP+GTP+X+XR+UA+G+GR
XMP aminase	GMP+GDP+GTP+G+GR
GMP kinase	GDP+GTP
GDP kinase	GTP
AMP dephosphorylase	A+AR
AR phosphorylase	A
IMP dephosphorylase	H+HR
HR phosphorylase	H
XMP dephosphorylase	X+XR+UA
XR phosphorylase	X+UA
X oxidase	UA
GMP dephosphorylase	G+GR
GR phosphorylase	G

Table 4B. Additional parameters of ATP catabolism.

Parameter	Function
AMP dephosphorylase/ AMP deaminase	(A+AR)÷(IMP+XMP+GMP+GDP+GTP+H+HR+ G+GR+X+XR+UA)
IMP dephosphorylase/ IMP dehydrogenase	(H+HR)÷(XMP+GMP+GDP+GTP+G+GR+X+ XR+UA)
XMP dephosphorylase/ XMP aminase	(X+XR+UA)÷(GMP+GDP+GTP+G+GR)
AMP dephosphorylase/ IMP dephosphorylase	(A+AR)÷(H+HR)

```
    ∇ AMETAB;V;RV;I
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'SAMPLE   GTP   ATP   GDP   ADP   GMP   XMP   IMP   AMP   NAD   A   AR   H   HR   G   GR   X   XR   UA   N.A.'
[3]  LABEL1:→(1=⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],((+/1+V)-V[11]),(+/V[3 5 10 20]),(+/V[3 10 20]),V[20],(+/V[2 4 6 7 8 13 14 15 16 17 18 19])
[5]   RV←RV,(+/V[2 4 6 7 15 16 17 18 19]),(+/V[2 4 6 15 16]),(+/V[2 4]),V[2],V[12],(+/V[13 14]),(+/V[17 18 19])
[6]   RV←RV,(+/V[15 16]),V[13],(+/V[17 19]),V[15],V[19],(+/V[3 5 9 10 20]),(V[3]÷+/V[5 9]),(+/V[2 4 6 7 8])
[7]   RV←RV,(V[2]÷+/V[4 6]),((+/V[3 5 9 10 20])÷(+/V[2 4 6 7 8]))
[8]   RV←RV,((+/V[3 5 10 20])÷(+/V[2 4 6 7 8 13 14 15 16 17 18 19]))
[9]   RV←RV,(+/V[12 13 14 15 16 17 18 19]),((+/V[12 13 14 15 16 17 18 19])÷((+/1+V)-V[11]))
[10]  RV←RV,(V[12]÷+/V[3 5 9 10 12 20]),((+/V[13 14])÷(+/V[2 4 6 7 8 13 14 15 16 17 18 19]))
[11]  RV←RV,((+/V[17 18 19])÷(+/V[2 4 6 7 17 18 19]))
[12]  RV←RV,((+/V[15 16])÷(+/V[2 4 6 15 16])),(V[13]÷+/V[13 14]),((+/V[17 19])÷(+/V[17 18 19]))
[13]  RV←RV,(V[15]÷+/V[15 16]),(V[20]÷+/V[3 10 20])
[14]  →LABEL1,I←I+1
[15] LABEL2:(1 0 ,(17⍴1),0,(16⍴1))\[1] ADAT,(10 3) DFT RV←⍉(I,34)⍴RV
    ∇
```

```
    ∇ RMETAB;V;RV;I
[1]    'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍬⋄I←0
[2]    'SAMPLE GTP ATP GDP ADP GMP XMP IMP AMP NAD A AR E HR G GR X XR UA AN.A. GN.A. RNA'
[3]   LABEL1:→(1=⍴,V←⎕)/LABEL2
[4]    RV←RV,V[1],((+/1↓V)-(+/V[13 17 19 20 21])),(+/V[3 5 9 10 11 12 20]),(+/V[3 5 10 20]),(+/V[3 10 20])
[5]    RV←RV,(+/V[2 4 6 7 15 16 18 21]),(+/V[2 4 6 15 16 21]),(+/V[2 4 21]),(+/V[2 21]),V[22],(+/V[11 12])
[6]    RV←RV,V[14],V[18],(+/V[15 16]),V[11],V[15],(+/V[17 19]),(+/V[3 5 9 10 20]),(V[3]÷+/V[5 9])
[7]    RV←RV,(+/V[2 4 6 7 21]),(V[2]÷+/V[4 6]),((+/V[3 5 9 10 20])÷(+/V[2 4 6 7 21]))
[8]    RV←RV,((+/V[3 5 9 10 11 12 20])÷(+/V[2 4 6 7 15 16 18 21]))
[9]    RV←RV,(((+/1↓V)-(+/V[13 17 19 20 21]))÷(+/V[17 19])),(V[22]÷+/V[2 3 10 22])
[10]   RV←RV,(V[20]÷+/V[3 10 20]),(V[21]÷+/V[2 21]),(+/V[11 12 14 15 16 18])
[11]   RV←RV,((+/V[11 12 14 15 16 18])÷((+/1↓V)-(+/V[13 17 19 20 21])))
[12]   RV←RV,((+/V[11 12])÷(+/V[3 5 9 10 11 12 20])),(V[14]÷((+/1↓V)-(+/V[13 17 19 20 21])))
[13]   RV←RV,(V[18]÷+/V[2 4 6 7 15 16 18 21]),((+/V[15 16])÷(+/V[2 4 6 15 16 21]))
[14]   RV←RV,(V[11]÷+/V[11 12]),(V[15]÷+/V[15 16])
[15]   →LABEL1,I←I+1
[16]  LABEL2:(1 0 ,(16⍴1),0,(18⍴1))\[1] RDAT,(10 3) DFT RV←⍉(I,35)⍴RV
    ∇
```

```
    ∇ GMETAB;V;RV;I
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'SAMPLE  GTP  ATP  GDP  ADP  GMP  XMP  IMP  AMP  NAD  A  AR  H  HR  G  GR  X  XR  UA  N.A.'
[3]  LABEL1:→(1=⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],((+/1+V)-(+/V[15 17 19])),(+/V[2 4 20]),(+/V[2 20]),V[20]
[5]   RV←RV,(+/V[3 5 7 8 9 10 11 12 13 14 18]),(+/V[7 18]),(+/V[3 5 9 10 11 12]),(+/V[3 5 10])
[6]   RV←RV,(+/V[3 10]),(+/V[11 12]),(+/V[13 14]),V[18],V[16],V[11],V[13],(+/V[17 19]),V[19]
[7]   RV←RV,(+/V[2 4 6 20]),(V[2]÷+/V[4 6]),(+/V[3 5 7 8 9 10]),(V[3]÷+/V[5 9])
[8]   RV←RV,((+/V[2 4 6 20])÷(+/V[3 5 7 8 9 10]))
[9]   RV←RV,((+/V[2 4 20])÷(+/V[3 5 7 8 9 10 11 12 13 14 18]))
[10]  RV←RV,(((+/1+V)-(+/V[15 17 19]))÷(+/V[17 19])),(+/V[11 12 13 14 16 18])
[11]  RV←RV,((+/V[11 12 13 14 16 18])÷((+/1+V)-(+/V[15 17 19])))
[12]  RV←RV,((+/V[11 12])÷(+/V[3 5 9 10 11 12])),((+/V[13 14])÷(+/V[3 5 7 8 9 10 11 12 13 14 18]))
[13]  RV←RV,(V[18]÷+/V[7 18]),(V[16]÷((+/1+V)-(+/V[15 17 19]))),(V[11]÷+/V[11 12])
[14]  RV←RV,(V[13]÷+/V[13 14]),(V[20]÷+/V[2 20])
[15]  →LABEL1,I←I+1
[16] LABEL2:(1 0 ,(17⍴1),0,(18⍴1))\[1] GDAT,(10 3) DFT RV←⍉(I,34)⍴RV
    ∇
```

Hollerith matrices ADAT, HDAT and GDAT for programs AMETAB, HMETAB and GMETAB

ADAT	HDAT	GDAT
SAMPLE NUMBER	SAMPLE NUMBER	SAMPLE NUMBER
APRTASE	HGPRTASE	GPRTASE
AMP KINASE	AMPS SYNTH.+LYASE	GMP KINASE
ADP KINASE	AMP KINASE	GDP KINASE
RNA POLYMERASE	ADP KINASE	RNA POLYMERASE
AMP DEAMINASE	IMP DEHYDROGENASE	GMP REDUCTASE
IMP DEHYDROGENASE	XMP AMINASE	IMP DEHYDROGENASE
XMP AMINASE	GMP KINASE	AMPS SYNTH.+LYASE
GMP KINASE	GDP KINASE	AMP KINASE
GDP KINASE	RNA POLYMERASE	ADP KINASE
AMP DEPHOSPHORYLASE	AMP DEPHOSPHORYLASE	AMP DEPHOSPHORYLASE
IMP DEPHOSPHORYLASE	IMP DEPHOSPHORYLASE	IMP DEPHOSPHORYLASE
XMP DEPHOSPHORYLASE	XMP DEPHOSPHORYLASE	XMP DEPHOSPHORYLASE
GMP DEPHOSPHORYLASE	GMP DEPHOSPHORYLASE	GMP DEPHOSPHORYLASE
HR PHOSPHORYLASE	AR PHOSPHORYLASE	AR PHOSPHORYLASE
XR PHOSPHORYLASE	GR PHOSPHORYLASE	HR PHOSPHORYLASE
GR PHOSPHORYLASE	XANTHINE OXIDASE	GUANASE
XANTHINE OXIDASE	TOTAL 'A' NUCLEOTIDES	XANTHINE OXIDASE
TOTAL 'A' NUCLEOTIDES	A ENERGY STATUS	TOTAL 'G' NUCLEOTIDES
A ENERGY STATUS	TOTAL 'G' NUCLEOTIDES	G ENERGY STATUS
TOTAL 'G' NUCLEOTIDES	G ENERGY STATUS	TOTAL 'A' NUCLEOTIDES
G ENERGY STATUS	A/G NUCLEOTIDE RATIO	A ENERGY STATUS
A/G NUCLEOTIDE RATIO	AMPS SYNTH./IMP DEHYD.	G/A NUCLEOTIDE RATIO
AMP KINASE/AMP DEAMINASE	HGPRTASE/X OXIDASE	GMP KINASE/GMP REDUCTASE
TOTAL TIDE CATABOLISM	NA./TOTAL NTP	GPRTASE/GUANASE
FRACTION TIDE CATAB.	A N.A./ATP SYNTHESIS	TOTAL 'TIDE CATABOLISM
FRACTION AMP CATAB.	G N.A./GTP SYNTHESIS	FRACTION 'TIDE CATAB.
FRACTION IMP CATAB.	TOTAL 'TIDE CATABOLISM	FRACTION AMP CATAB.
FRACTION XMP CATAB.	FRACTION 'TIDE CATAB.	FRACTION IMP CATAB.
FRACTION GMP CATAB.	FRACTION AMP CATAB.	FRACTION XMP CATAB.
FRACTION HR CATAB.	FRACTION IMP CATAB.	FRACTION GMP CATAB.
FRACTION XP CATAB.	FRACTION XMP CATAB.	FRACTION AR CATAB.
FRACTION GR CATAB.	FRACTION GMP CATAB.	FRACTION HR CATAB.
FRACTION ATP INCOR. N.A.	FRACTION AR CATAB.	FRACTION GTP INCOR. N.A.
	FRACTION GR CATAB.	

The program HSCREEN written in the APL language for the APL/360 Terminal System calculates apparent rates, fractional inhibition and percent inhibition of enzymes and parameters represented by the variable DATA. Input of each set of data for nucleotides is requested in a specified order and control data must be entered first and assigned the identification number (C.R.U. Number) 1.

```
DATA

C.R.U. NUMBER
HGPRTASE
AMPS SYNTH.+LYASE
AMP KINASE
ADP KINASE
IMP DEHYDROGENASE
GMP SYNTHETASE
GMP KINASE
GDP KINASE
TOTAL A NUCLEOTIDES
A ENERGY STATUS
TOTAL G NUCLEOTIDES
G ENERGY STATUS
A/G NUCL. RATIO
```

```
    ∇ HSCREEN;V;RV;I
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'CRU   GTP   ATP   GDP   ADP   GMP   XMP   IMP   AMP   NAD'
[3]  LABEL1:→(1=⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],(+/1↓V),(+/V[3 5 9 10]),(+/V[3 5 10]),(+/V[3 10]),(+/V[2 4 6 7]),(+/V[2 4 6]),(+/V[2 4]),V[2]
[5]   RV←RV,(+/V[3 5 9 10]),(V[3]÷+/V[5 9]),(+/V[2 4 6 7]),(V[2]÷+/V[4 6]),((+/V[3 5 9 10])÷(+/V[2 4 6 7]))
[6]   →LABEL1,I←I+1
[7]  LABEL2:(1 0 ,13⍴1)\[1] DATA,(10 3) DFT RV←⍉(I,14)⍴RV
[8]   '  '
[9]   'EXPRESSED AS FRACTION OF THE CONTROL VALUE:'
[10]  '  '
[11]  (1 0 ,13⍴1)\[1] DATA,(11 5) DFT RV←(0 1)↓RV÷⍉(⍴⍉RV)⍴RV[;1]
[12]  '  '
[13]  'PERCENTAGE INHIBITION:'
[14]  '  '
[15]  V←(9,I)⍴RV[1;],100×1-((,RV)[I+⍳8×I])÷((I←I-1)⍴1),(,(1 0)+(¯10 0)+RV),RV[2;],,(5 0)+(¯6 0)+RV
[16]  (1 0 ,8⍴1)\[1]((¯5 0)+DATA),(11 1) DFT V
    ∇
```

```
    ∇ HSCREENNA;V;RV;I
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'SAMPLE   GTP   ATP   GDP   ADP   GMP   XMP   IMP   AMP   NAD   ANA   GNA'
[3]  LABEL1:→(1=ρ,V←⍞)/LABEL2
[4]   RV←RV,V[1],(+/1↓V),(+/V[3 5 9 10 11]),(+/V[3 5 10 11]),(+/V[3 10 11]),V[11],(+/V[2 4 6 7 12])
[5]   RV←RV,(+/V[2 4 6 12]),(+/V[2 4 12]),(+/V[2 12]),V[12],((+/V[3 5 9 10 11])÷(+/V[2 4 6 7 12]))
[6]   RV←RV,(V[3]÷+/V[5 9]),(V[2]÷+/V[4 6]),(V[11]÷+/V[3 10 11]),(V[12]÷+/V[2 12]),(+/V[11 12])
[7]   →LABEL1,I←I+1
[8]  LABEL2:(1 0 ,(16ρ1))\[1] HTD,(10 3) DFT RV←⍉(I,17)ρRV
[9]   '  '
[10]  'EXPRESSED AS A FRACTION OF THE CONTROL:'
[11]  '  '
[12]  (1 0 ,(16ρ1))\[1] HTD,(11 5) DFT RV←(0 1)↓RV÷⍉(ρ⍉RV)ρRV[;1]
    ∇
```

```
      HTD

 SAMPLE NUMBER
 HGPRTASE
 AMPS SYNTH. + LYASE
 AMP KINASE
 ATP KINASE
 N.A. SYNTHESIS (ATP)
 IMP DEHYDROGENASE
XMP AMINASE
 GMP KINASE
 GDP KINASE
 N.A. SYNTHESIS (GTP)
 AMPS SYNTH./IMP DEHYD.
 A ENERGY STATUS
 G ENERGY STATUS
 A N.A./ADP KINASE
 G N.A./GDP KINASE
 TOTAL N.A.
```

```
    ∇ ASCREEN;V;RV;I;AV
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'SAMPLE   GTP   ATP   GDP   ADP   GMP   XMP   IMP   AMP   NAD   A   AR   H   HR   G   GR   X   XR   UA   N.A.'
[3]  LABEL1:→(1=⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],((+/1+V)-V[11]),(+/V[3 5 10 20]),(+/V[3 10 20]),V[20],(+/V[2 4 6 7 8 13 14 15 16 17 18 19])
[5]   RV←RV,(+/V[2 4 6 7 15 16 17 18 19]),(+/V[2 4 6 15 16]),(+/V[2 4]),V[2],V[12],(+/V[13 14]),(+/V[17 18 19])
[6]   RV←RV,(+/V[15 16]),V[13],(+/V[17 19]),V[15],V[19],(+/V[3 5 9 10 20]),(V[3]+÷+/V[5 9]),(+/V[2 4 6 7 8])
[7]   RV←RV,(V[2]+÷+/V[4 6]),((+/V[3 5 9 10 20])÷(+/V[2 4 6 7 8]))
[8]   RV←RV,((+/V[3 5 10 20])÷(+/V[2 4 6 7 8 13 14 15 16 17 18 19]))
[9]   RV←RV,(+/V[12 13 14 15 16 17 18 19]),((+/V[12 13 14 15 16 17 18 19])÷((+/1+V)-V[11]))
[10]  RV←RV,(V[12]+÷+/V[3 5 9 10 12 20]),((+/V[13 14])÷(+/V[2 4 6 7 8 13 14 15 16 17 18 19]))
[11]  RV←RV,((+/V[17 18 19])÷(+/V[2 4 6 7 17 18 19]))
[12]  RV←RV,((+/V[15 16])÷(+/V[2 4 6 15 16])),(V[13]+÷+/V[13 14]),((+/V[17 19])÷(+/V[17 18 19]))
[13]  RV←RV,(V[15]+÷+/V[15 16]),(V[20]+÷+/V[3 10 20])
[14]  →LABEL1,I←I+1
[15] LABEL2:(1 0 ,(17⍴1),0,(16⍴1))\[1] ADAT,(10 3) DFT RV←⍉(I,34)⍴RV
[16]  ' '
[17]  'EXPRESSED AS A FRACTION OF THE CONTROL:'
[18]  ' '
[19]  (1 0 ,(17⍴1),0,(16⍴1))\[1] ADAT,(11 5) DFT RV←(0 1)↓RV÷⍉(⍴⍉RV)⍴RV[;1]
[20]  ' '
[21]  'PERCENT INHIBITION:'
[22]  ' '
[23]  AV←((I←I-1)⍴1),(,(1 0)+(¯30 0)↓RV),RV[2;],,(5 0)+(¯25 0)↓RV
[24]  AV←AV,(RV[2;],,(10 0)+(¯24 0)↓RV),(RV[6;],,(11 0)+(¯23 0)↓RV),(RV[7;],,(12 0)+(¯22 0)↓RV)
[25]  AV←AV,(RV[8;],,(13 0)+(¯21 0)↓RV),(RV[12;],,(14 0)+(¯20 0)↓RV),(RV[13;],,(15 0)+(¯19 0)↓RV)
[26]  AV←AV,(RV[14;],,(16 0)+(¯18 0)↓RV),(RV[16;],,(17 0)+(¯17 0)↓RV)
[27]  V←(18,I)⍴RV[1;],100×1-(((,RV)[I+⍳17×I])÷AV)
[28]  (1 0 ,17⍴1)\[1]((¯16 0)↓ADAT),(11 1) DFT V
    ∇
```

```
    ∇ ATPCATAB;V;RV;R
[1]   'ENTER DATA IN THE FOLLOWING ORDER. WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'MIN  GTP  ATP  GDP  ADP  GMP  XMP  IMP  AMP  NAD  A  AR  H  HP  G  GR  X  XP  UA'
[3]  LABEL1:→(1=⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],((+/1↓V)-(+/V[3 10])),((+/1↓V)-(+/V[3 5 10])),((+/1↓V)-(+/V[3 5 9 10 11 12]))
[5]   RV←RV,((+/1↓V)-(+/V[3 5 8 9 10 11 12 13 14])),(+/V[2 4 6 15 16]),(+/V[2 4]),V[2]
[6]   RV←RV,(+/V[11 12]),V[11],(+/V[13 14]),V[14],(+/V[17 18 19]),(+/V[18 19]),V[19],(+/V[15 16]),V[15]
[7]   RV←RV,(V[3]÷+/V[3 5 9]),(V[3]÷+/V[5 9]),(V[2]÷+/V[4 6])
[8]   →LABEL1,I←I+1
[9]  LABEL2:(1 0 ,(16⍴1),0,(3⍴1))\[1] ATP,(10 3) DFT RV←⍉(I,20)⍴RV
[10]  ' '
[11]  'CORRECTED FOR ZERO MINUTE APPARENT RATE'
[12]  ' '
[13]  (1 0 ,16⍴1)\[1]((¯3 0)↓ATP),(10 3) DFT RV←(¯3 1)↓RV-⍉(⍴⍉RV)⍴RV[;1]
[14]  ' '
[15]  'RATIOS'
[16]  ' '
[17]  R←RV[1;],(RV[9;]÷RV[4;]),(RV[11;]÷RV[5;]),(RV[13;]÷RV[6;]),(RV[16;]÷RV[7;]),(RV[9;]÷RV[11;])
[18]  (1 0 ,(5⍴1))\[1] RAT,(10 3) DFT P←(6,(I-1))⍴R
    ∇
```

ATP

```
MINUTES
ATP DEPHOSPHORYLASE
ADP DEPHOSPHORYLASE
AMP DEAMINASE
IMP DEHYDROGENASE
XMP AMINASE
GMP KINASE
GDP KINASE
AMP DEPHOSPHORYLASE
AR PHOSPHORYLASE
IMP DEPHOSPHORYLASE
HP PHOSPHOPYLASE
XMP DEPHOSPHORYLASE
XP PHOSPHORYLASE
X OXIDASE
GMP DEPHOSPHORYLASE
GR PHOSPHORYLASE
FRACTION A 'TIDES ATP
A ENERGY STATUS
G ENERGY STATUS
```

RAT

```
TIME
AMP DEPHOS TO AMP DEAM
IMP DEPHOS TO IMP DEHYD
XMP DEPHOS TO XMP AMIN
GMP DEPHOS TO GMP KINASE
AMP DEPHOS TO IMP DEPHOS
```

CHAPTER 5

PURINE NUCLEOTIDE SYNTHESIS IN SYNCHRONIZED LYMPHOMA L5178Y CELLS

INTRODUCTION

A delayed lethal effect of 6-mercaptopurine on cultured mouse lymphoma L5178Y cells has recently been reported, and it was concluded that the action of 6-mercaptopurine is dependant on macromolecular synthesis (1). Consistent with this suggestion is the observation that 6-mercaptopurine exhibits its toxicity primarily during the S-phase of division of synchronized lymphoma L5178Y cells (A. R. P. Paterson, personal communication). However, it was of interest to examine the possibility that this cell cycle specificity of 6-mercaptopurine might also be related to variations in the activities of enzymes of purine metabolism or of concentrations of purine nucleotides through the cell cycle. As there was very little information available regarding such variations in intact mammalian cells, various aspects of purine metabolism were therefore examined using synchronized lymphoma L5178Y cells.

MATERIALS AND METHODS

Cell cultures

Mouse lymphoma L5178Y cells were routinely grown in suspension cultures with Fischer's medium (Grand Island

Biological Co., Grand Island, N.Y.) supplemented with 10% horse serum, streptomycin (100 µg/ml) and penicillin (100 units/ml) (2). Cell numbers were determined with Coulter Model F or Model B electronic particle counters. Synchronous L5178Y cultures were provided by S. C. Kim and A. R. P. Paterson. The cells were synchronized by a 5 hr exposure to 2 mM thymidine followed by a 5 hr exposure to 0.02 µg/ml colcemid (Grand Island Biological Co.). This procedure is similar to that described by Doida and Okada (3). After resuspension in fresh medium, between 70 and 80% of the cells present underwent synchronous division (Fig. 1).

Incubation with purine bases

Duplicate 2.5 ml culture samples were transferred to stoppered 16 x 100 mm tubes and maintained at 37° for 20 min; radioactive purine bases were then added. Adenine-8-^{14}C, 39.3 mCi/mmole, guanine-8-^{14}C, 41.8 mCi/mmole and hypoxanthine-8-^{14}C, 49.4 mCi/mmole, were obtained from Schwarz BioResearch. Incubations were terminated 30 min later by pouring the cultures into prechilled centrifuge tubes, centrifuging down the cells in a clinical centrifuge, and extracting the cells with 0.010 ml of 0.4 M perchloric acid. After neutralization with 0.010 ml of 0.4 M KOH, the entire sample was spotted on polyethyleneimine-cellulose

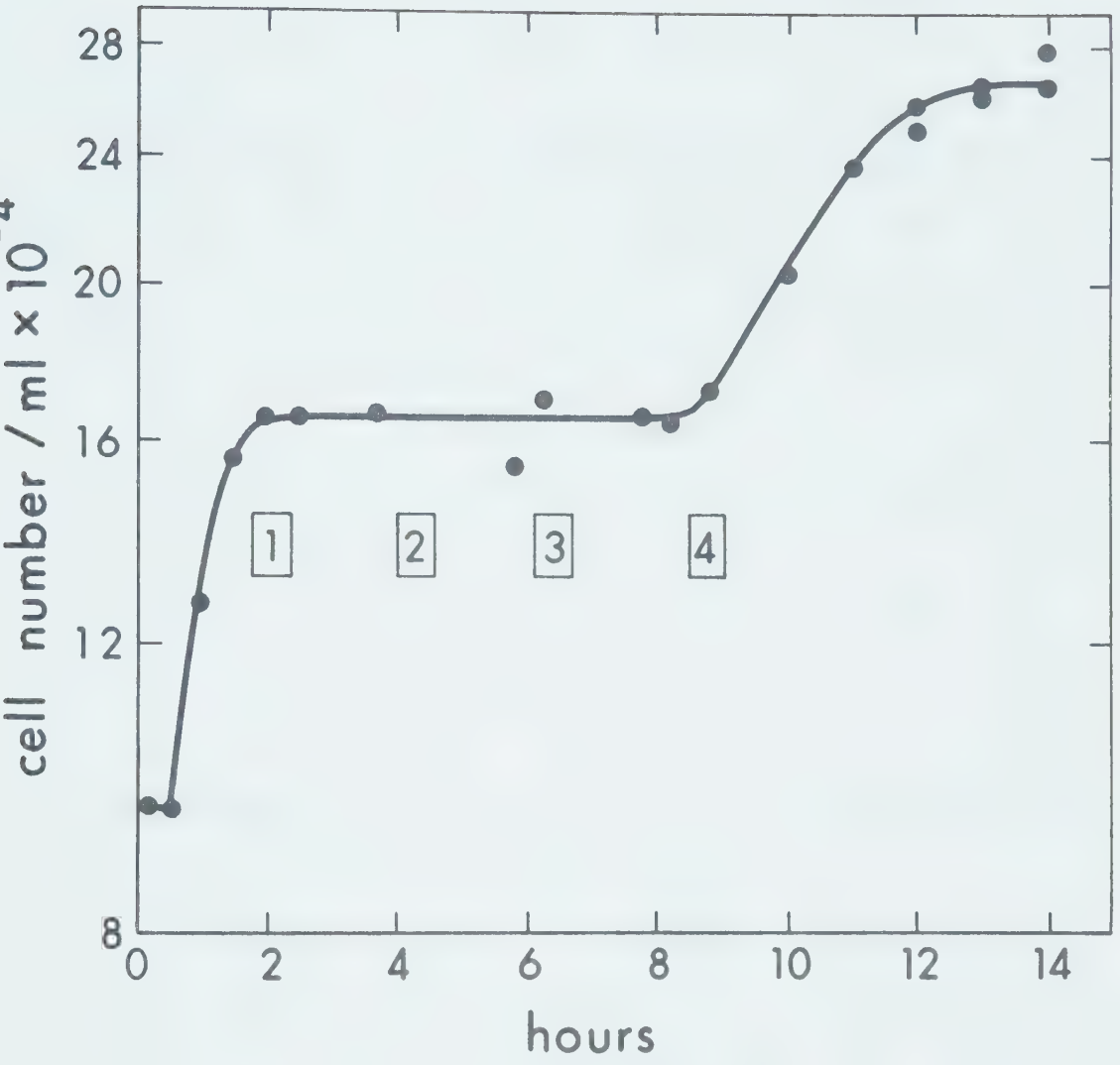

Figure 1. Periods of study of synchronized lymphoma L5178Y cells.

The metabolism of radioactive purine bases was determined during the four 30 min periods indicated.

plates (Polygram Cel 300 PEI, Brinkman Instruments).

Chromatography

The purine ribo- and deoxyribonucleotides were separated by two-dimensional chromatography on PEI-cellulose thin layer sheets by a modification of the methods of Crabtree and Henderson (4) and Cashel and Kalbacher (5).

Samples of cell extracts plus carriers are placed on the origin 2 cm from the bottom of the sheet in the first dimension and 2.5 cm from the bottom of the sheet in the second dimension. A wick of Whatman 3MM filter paper is attached with staples to the top of the sheet in the direction of the first dimension. The chromatogram is developed with methanol/water (1/1) for 14 to 16 hr to wash bases and nucleosides onto the wick and then dried.

For chromatography in the first dimension the plate was developed to Front 1A with 1.8 M ammonium formate, 2% boric acid, pH 7; and then developed until the leading spot was near the wick (Front 2A) with 3.3 M ammonium formate, 4.2% boric acid, pH 7. The wick was removed and the chromatogram immediatedly immersed in a methanol bath for 12 minutes.

After the sheets were dried, chromatography was begun in the second dimension. The plate was developed to Front 1B with 0.5 M sodium formate buffer, pH 3.4; to Front

2B with 2.0 M sodium formate buffer, pH 3.4; and then to Front 3B with 4.0 M sodium formate buffer, pH 3.4.

In the first dimension Front 1A is 8.0 cm above the origin and Front 2A is approximately 17.5 cm above the origin. In the second dimension Front 1B is 2.5 cm above the origin, Front 2B is 8 cm above the origin and Front 3B approximately 17 cm above the origin.

A typical chromatogram showing the resolution of a mixture of authentic nucleotides is shown in Figure 2.

RESULTS

The labeling of ATP and GTP from radioactive bases in asynchronous lymphoma cells was linear until 30 min. Figure 3 shows the relationship between total nucleotide synthesis and purine base concentration for exponentially growing lymphoma L5178Y cells. Nucleotide synthesis from hypoxanthine and guanine was near maximal at 40 μM while that for adenine was still increasing at 50 μM. Because adenine is markedly toxic at concentrations above 20 μM (6), this concentration of all three purines was chosen for the study of synchronous cells.

The metabolism of the purine bases in the culture medium alone was examined by incubating either 20 μM adenine,

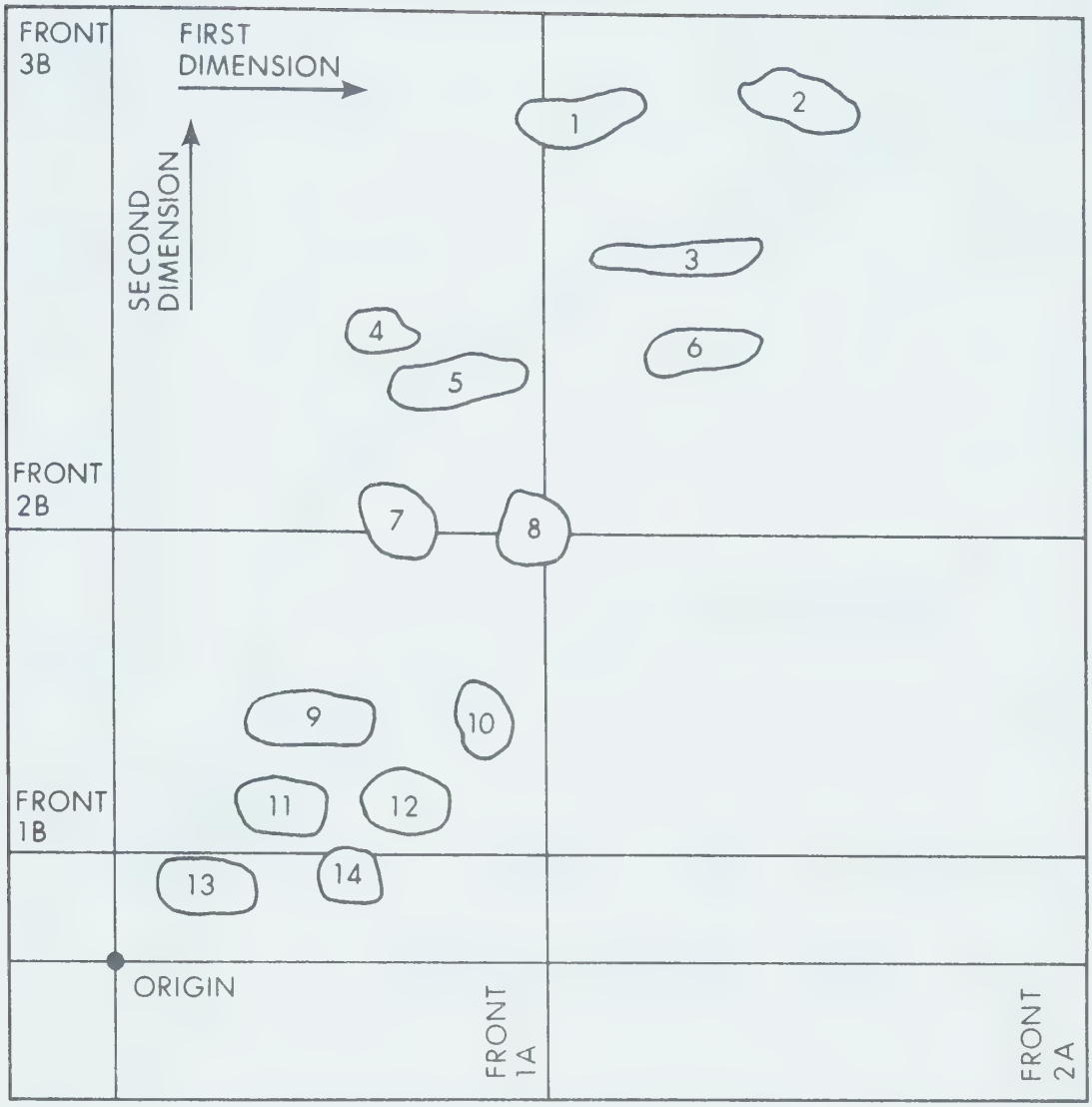

Figure 2. Two-dimensional chromatography of purine ribo- and deoxyribonucleotides on PEI-cellulose.

Ultraviolet absorbing areas are: 1, AMP; 2, dAMP; 3, IMP; 4, XMP; 5, GMP; 6, dGMP; 7, ADP; 8, dADP; 9, GDP; 10, dGDP; 11, ATP; 12, dATP; 13, GTP; 14, dGTP.

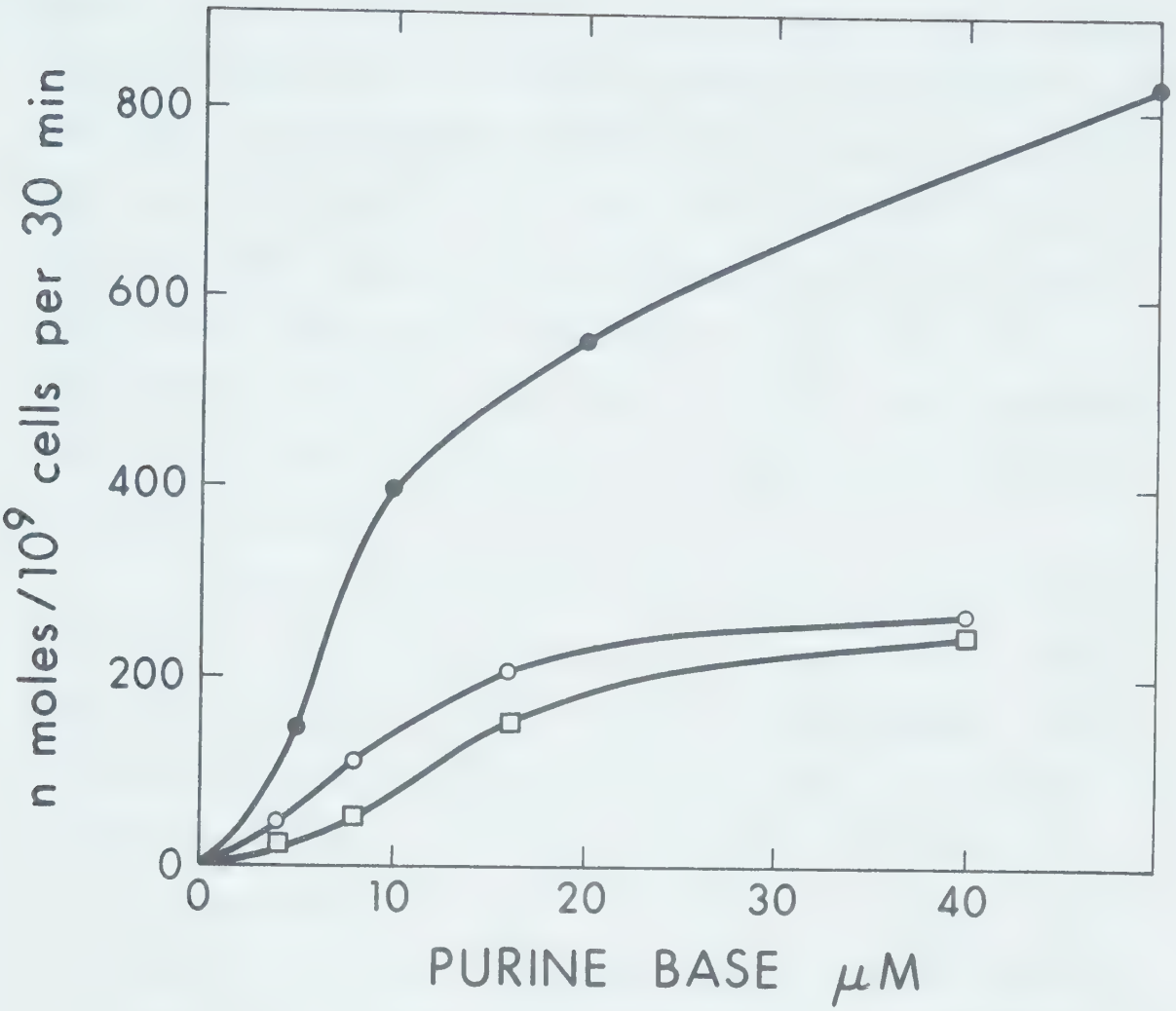

Figure 3. Acid-soluble nucleotide synthesis in asynchronous lymphoma L5178Y cells.

Incubation time: 30 min
Cell concentration: 141,800 cells per ml.
Precursors: (●) adenine-^{14}C
(o) hypoxanthine-^{14}C
(□) guanine-^{14}C

guanine or hypoxanthine for 30 min with medium minus cells. The purine bases were isolated by column chromatography on Dowex-50, and subsequent two-dimensional chromatography on cellulose thin layers. In 30 min 80% of the guanine originally present was converted to xanthine, 3% of the hypoxanthine was converted to xanthine, but there was no detectable metabolism of the adenine. The guanine deaminase activity has been attributed to the horse serum present in the culture medium.

Samples from a synchronous lymphoma L5178Y culture were taken at the four times shown on Figure 1 which corresponded to periods in the G_1-phase, early S-phase, late S-phase, and G_2-phase as indicated by thymidine labeling experiments (A. R. P. Paterson, personal communication). The G_1-phase sample was taken just after the increase in cell number had reached a plateau, which was approximately 2 hr after the release of the colcemid block. The subsequent samples were taken at 2 hr intervals.

Total nucleotide synthesis from each purine base during the 4 periods is shown in Figure 4. Nucleotide synthesis from adenine increased to a maximum during the late S-phase period and then declined in the G_2-phase period. Extents of nucleotide synthesis from hypoxanthine and guanine were parallel, but did not exhibit the magnitude of variation seen with adenine.

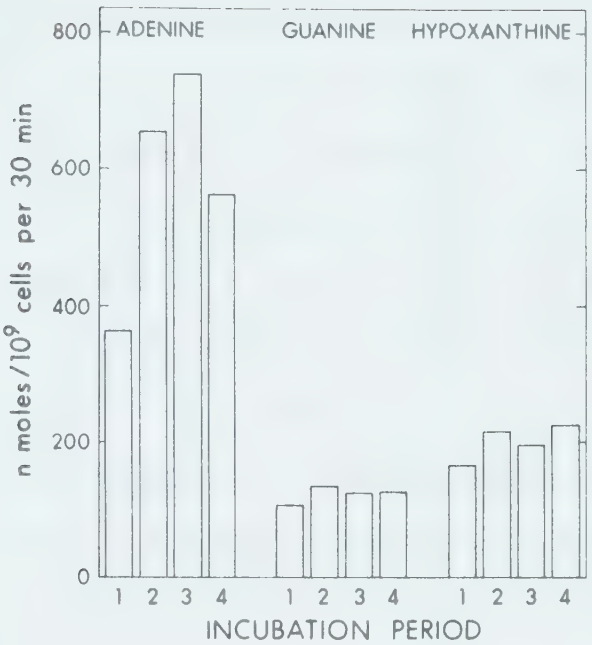

Figure 4. Acid-soluble nucleotide synthesis in synchronous lymphoma L5178Y cells.

Precursors: adenine-^{14}C (20 μM)
guanine-^{14}C (20 μM)
hypoxanthine-^{14}C (20 μM)

Numbers under each bar refer to periods of study shown in Figure 1.

The synthesis of individual nucleotides, expressed in nmoles per 10^9 cells, for the four 30 min labeling periods is presented in Table 1. Most of the acid-soluble radioactivity accumulated in the ribonucleoside triphosphates, namely ATP and GTP. The level of incorporation into the nucleoside monophosphates and diphosphates remained low, which indicates that uptake and conversion of bases to nucleotides was rate-limiting for ribonucleoside triphosphate synthesis. The radioactivity found in dATP and dGTP was generally 1 to 2% of that found in ATP and GTP.

By assuming a simplified uni-directional pathway of metabolism for the synthesis of nucleoside triphosphates from each purine base, apparent activities may be calculated for several of the reactions of purine metabolism (Chapters 3 and 4). The apparent activity for a given step is taken to be represented by the sum of the radioactivity in all ribonucleotide and deoxyribonucleotide components which occur further along the pathway from the step in question. These results are shown in Table 2 and the ratios of processes having the common substrates AMP, GMP or IMP are also given. The apparent activity of the phosphoribosyltransferases is equal to total nucleotide synthesis. The apparent activity of AMP deaminase increased from the G_1-phase period through the G_2-phase period, and the lowest AMP kinase/AMP deaminase ratio was in the G_2-phase period. The apparent activity of

TABLE 1. Nucleotides synthesized from purine bases.

Synchronized L5178Y cells were incubated during the four 30 min periods of study shown in Figure 1, with 20 μM adenine-^{14}C, guanine-^{14}C or hypoxanthine-^{14}C. Radioactivity measured in nucleotides is expressed as nmoles formed in 30 min per 10^9 cells and is the average of two determinations.

	Adenine				Hypoxanthine				Guanine*			
Nucleotide	1	2	3	4	1	2	3	4	1	2	3	4
Total	363	659	734	557	165	216	196	224	107	137	126	128
ATP	286	545	635	465	94.7	126	110	131	1.7	14.6	2.6	2.2
ADP	46.4	66.7	56.9	53.0	12.1	22.2	22.3	23.1	0.5	3.0	1.3	0.7
AMP	15.1	15.5	8.9	7.7	1.8	4.1	9.9	6.2	---	1.5	1.1	0.2
dATP	3.9	7.6	11.7	3.9	0.7	1.4	2.8	0.6				
dADP	---†	0.3	0.3	0.1	0.2	0.1	0.1	0.3				
dAMP	1.5	1.4	0.3	0.8	0.3	1.2	---	0.3				
GTP	6.7	15.3	17.0	11.2	47.0	49.2	38.5	46.8	81.9	102	104	104
GDP	1.7	4.0	1.3	4.5	6.7	8.3	8.6	9.0	17.6	12.4	12.6	16.8
GMP	---	---	---	---	0.3	1.6	1.7	1.4	3.8	0.7	3.5	3.0
dGTP	0.5	1.1	1.3	0.6	0.5	0.9	0.6	0.6				
dGDP	---	---	---	---	---	---	---	---				
dGMP	---	---	---	---	0.1	0.2	0.1	0.1				
IMP	1.6	2.0	1.5	10.5	0.7	0.3	0.7	4.6	0.7	1.7	1.2	0.3
XMP	---	---	---	---	---	---	0.3	0.3	0.8	0.8	---	1.0

* Deoxyribonucleotides not determined with guanine labelling

† No detectable levels of these nucleotides.

Table 2. Apparent enzyme activities during cell cycle.

Parameter	Period of Study 1	2	3	4
	(nmoles/10^9 cells per 30 min)			
PRECURSOR: ADENINE				
Adenine phosphoribosyl-transferase	363	659	734	557
AMP kinase	338	621	704	523
AMP deaminase	11	22	21	27
PRECURSOR: HYPOXANTHINE				
Hypoxanthine phospho-ribosyltransferase	165	216	196	224
AMPS synthetase plus lyase	110	155	145	162
IMP dehydrogenase	55	60	50	58
PRECURSOR: GUANINE				
Guanine phosphoribosyl-transferase	107	137	126	128
GMP kinase	100	114	117	121
GMP reductase	4	22	6	4
RATIO OF APPARENT ACTIVITIES:				
AMP kinase/AMP deaminase	31	28	34	19
AMPS synthetase plus lyase/ IMP dehydrogenase	2.0	2.6	2.9	2.8
GMP kinase/GMP reductase	25	5	20	30

guanylate reductase was greatest in early S-phase, which also had the lowest GMP kinase/GMP reductase ratio. Variation in the apparent activities of AMPS synthetase plus lyase and IMP dehydrogenase was small and reflected the changes in hypoxanthine phosphoribosyltransferase.

Apparent activities of ribonucleotide reductase have not been calculated since the turnover of deoxyribonucleotide pools for DNA synthesis is rapid (7) and the pool sizes are small (8,9). As seen in Table 2, the deoxyribonucleotide levels were highest during the S-phase periods when either adenine or hypoxanthine was used as precursor.

DISCUSSION

The results of this study do not support the hypothesis that the S-phase toxicity of 6-mercaptopurine towards lymphoma L5178Y cells is due to variation in either the activity of some enzymes of purine metabolism or in ribonucleoside phosphate pool sizes through the cell cycle. It would thus appear more likely that the delayed lethal effect of 6MP on lymphoma L5178Y cells (1), is due to an effect on DNA rather than on RNA synthesis, or is related to the incorporation of some metabolite of 6MP into DNA.

The observation that there was no accumulation of

purine nucleoside monophosphates or diphosphates indicates that the uptake and conversion of purine bases to nucleoside monophosphates is the rate-limiting step for purine nucleoside triphosphate synthesis from purine bases. This rate-limiting monophosphate formation could conceivably be due to the amount of phosphoribosyltransferase activity present or to the availability of the second substrate, namely phosphoribosyl pyrophosphate (PRPP). Assays using cell-free extracts of lymphoma L5178Y have, however, shown that the rate of base uptake observed in these studies represents less than 5% of the total phosphoribosyltransferase activity (L. W. Brox, personal communication). Presumably then, it is the availability of PRPP that is the rate-limiting factor.

The increase in hypoxanthine-guanine phosphoribosyltransferase activity that is seen in Table 3 presumably parallels the increase in cell volume which occurs as the cells proceed through the cycle. The cause of the significant decrease in the adenine phosphoribosyltransferase activity observed during the G_2-phase is not known. It is possible that this reflects some change in PRPP metabolism and is observed only when adenine is used because of the greater nucleotide formation from this base.

As L5178Y cells normally grow in a medium lacking preformed purines, it is obvious that they can provide

their entire purine requirement by de novo synthesis. A minimal rate for purine de novo synthesis may then be estimated from the total purine content of the cell (RNA, DNA and acid soluble purine) and the doubling time. This calculation indicates that the rates of nucleotide formation from purine bases observed in these studies of synchronous cells are about 10-15% of the average rate of purine synthesis required for cell growth.

It is evident from the observed apparent activities of AMP deaminase and GMP reductase that routes of AMP and GMP metabolism are minor compared to phosphorylation by the AMP and GMP kinases. As might have been expected, the highest amounts of radioactive dATP and dGTP were observed during the 2 S-phase labeling periods. The radioactivity found in these deoxytriphosphates was generally 1 to 2% of that found in ATP and GTP, which is consistent with other reports on triphosphate levels in mammalian cells (8,9,10,11).

Mean cell volumes and concentrations of individual acid soluble nucleotides have also been measured in synchronous lymphoma L5178Y cells by L. W. Brox (personal communication). The concentrations of NAD, ADP, ATP and GTP remained essentially constant throughout the cell cycle and these results are consistent with a previous report of ATP concentrations in synchronous Chinese hamster

cells (12). The purine and pyrimidine monophosphates were present only in trace amounts. Thus it is apparent that any purine or pyrimidine antimetabolite which accumulates to any extent as the monophosphate may easily have a concentration equal to or greater than the naturally occuring monophosphates and hence may have profound effects on the overall metabolism of the cell.

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CHAPTER 6

EFFECTS OF ACTINOMYCIN D AND DAUNOMYCIN ON PURINE RIBONUCLEOTIDE METABOLISM IN EHRLICH ASCITES TUMOR CELLS *IN VITRO*

INTRODUCTION

Actinomycin D is well known to inhibit RNA synthesis (1) and daunomycin has been shown to inhibit the synthesis of both RNA and DNA in Ehrlich ascites tumor cells (2). However, actinomycin D appears also to affect other areas of metabolism. Thus, whereas Wheeler and Bennett (3) found that actinomycin D inhibited the incorporation of radioactive formate, glycine, hypoxanthine, adenine, and guanine, into RNA more than to DNA in Lactobacillus leichmannii, Ehrlich ascites tumor cells and H.Ep. #2 cells, incorporation into nucleic acid guanine was in some cases inhibited more than that into nucleic acid adenine. Experiments with L. leichmannii and H.Ep. #2 cells (3) indicated that actinomycin D also interferred with the synthesis of purine nucleotides de novo. These results led Wheeler and Bennett to postulate two additional sites of action for actinomycin D: one at the synthesis of purine nucleotides de novo and another in the utilization of guanine nucleotides for the synthesis of RNA.

These conclusions have been supported in subsequent studies. Thus Zbarsky (4) demonstrated that ^{14}C-formate incorporation into RNA and DNA in Ehrlich ascites tumor cells was inhibited by actinomycin D, with an especially marked inhibition of formate incorporation into

guanine of both nucleic acids. Jacoli and Zbarsky (5) also found that when Bacillus subtilis was grown on a medium containing radioactive glycine, actinomycin D treatment led to the accumulation of GTP, GDP, and IMP. In addition, Harbers and Müller (6) found that actinomycin D increased the amount of radioactivity in acid-soluble nucleotides synthesized from guanine in Ehrlich ascites tumor cells, whereas Lowy and Williams (7) concluded that actinomycin D did not inhibit guanine phosphoribosyltransferase or purine nucleoside phosphorylase activities in rabbit erythrocytes.

Other studies have been directed at the possible action of actinomycin D on oxidation-dependent biosynthetic processes. Thus, Honig and Rabinovitz (8,9) found that actinomycin D-induced inhibition of protein and sterol synthesis in Sarcoma 37 ascites cells could be relieved by the addition of glucose, although there was no effect on ATP concentrations (8).

The work reported here deals with the effects of actinomycin D and daunomycin on the synthesis and interconversion of purine nucleotides. A preliminary report of some of these findings has previously been presented (10).

MATERIALS AND METHODS

Sources of materials and methods of tumor cell preparation and incubation (Chapter 2), procedures for the separation and measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides (11), methods for the measurement of purine biosynthesis de novo (12) and of concentrations of phosphoribosyl pyrophosphate (13) and individual acid-soluble purine nucleotides (Chapter 2) have been reported previously. Actinomycin D and daunomycin were purchased from Calbiochem.

In experiments using adenine or guanine as labelled precursor, total acid-insoluble radioactivity was taken to be equivalent to nucleic acid adenine synthesis from adenine-^{14}C or nucleic acid guanine synthesis from guanine-^{14}C. Less than 2% of radioactive adenine nucleotides synthesized from adenine-^{14}C, were converted to guanine nucleotides, and less than 5% of radioactive guanine nucleotides synthesized from guanine-^{14}C were converted to adenine nucleotides. Acid-insoluble radioactivity was measured by adding 0.5 ml of cold 0.4 M perchloric acid to 100 µl of incubation mixture. These samples were then chilled and filtered on Whatman No. 3 filter paper discs which were successively washed with cold solutions of 2.5 ml of 0.4 M perchloric acid (twice), 2.5 ml of 0.3 M

trichloracetic acid (three times), and 2.5 ml of methanol (twice). Radioactivity on the dried discs was then measured (11).

In experiments using hypoxanthine as precursor, substantial amounts of radioactivity were found in both nucleic acid adenine and nucleic acid guanine. In this case 100 µl of incubation mixtures were transferred to 1.0 ml glass ampules containing 5 µl of 4.2 M perchloric acid, the acid insoluble precipitate was washed four times with 1.0 ml of 0.4 M perchloric acid, dried *in vacuo*, and to each ampule 100 µl of 1.0 N HCl was added. The ampules were sealed and placed in a boiling water bath for 60 min to hydrolyze the nucleic acids. Portions of the hydrolysates (50 µl) were chromatographed on Whatman No. 1 paper with carrier adenine and guanine for 5.5 hr (descending) with methanol, formic acid and water (70:15:5). Adenine and guanine spots having R_f values of 0.61 and 0.43 respectively, were cut out and their radioactivity measured.

A method for evaluating the effects of drugs on individual reactions of purine nucleotide metabolism in Ehrlich ascites tumor cells incubated with hypoxanthine-^{14}C has been reported (14). Here it is extended to reactions measured when cells are incubated with radioactive adenine or guanine. Results are presented in terms of the amount of radioactive substrate metabolized by the

different enzymes in a given period; other studies (11) have indicated that under these conditions radioisotope flow from purine bases to ATP and GTP is virtually equivalent to the observed changes in chemical composition of the triphosphate pools.

RESULTS AND DISCUSSION

Actinomycin D at a concentration of 5 µg/ml inhibited the incorporation of radioactive adenine, guanine and hypoxanthine into nucleic acids by 90% in Ehrlich ascites tumor cells, and half maximal inhibition was attained at approximately 1 µg/ml actinomycin D, as shown in Figure 1.

In preliminary experiments, incubation of cells with radioactive hypoxanthine and 5 µg/ml actinomycin D reduced the amount of radioactivity found in acid-soluble ATP and GTP by 30 and 70 percent, respectively. Since inhibition of RNA synthesis alone might be expected to cause an accumulation of radioactive ATP and GTP, this observed decrease suggested that actinomycin D affects purine metabolism at sites other than RNA synthesis. Actinomycin D, 5 µg/ml, was found also to reduce the radioactivity in adenine nucleotides synthesized from adenine-^{14}C

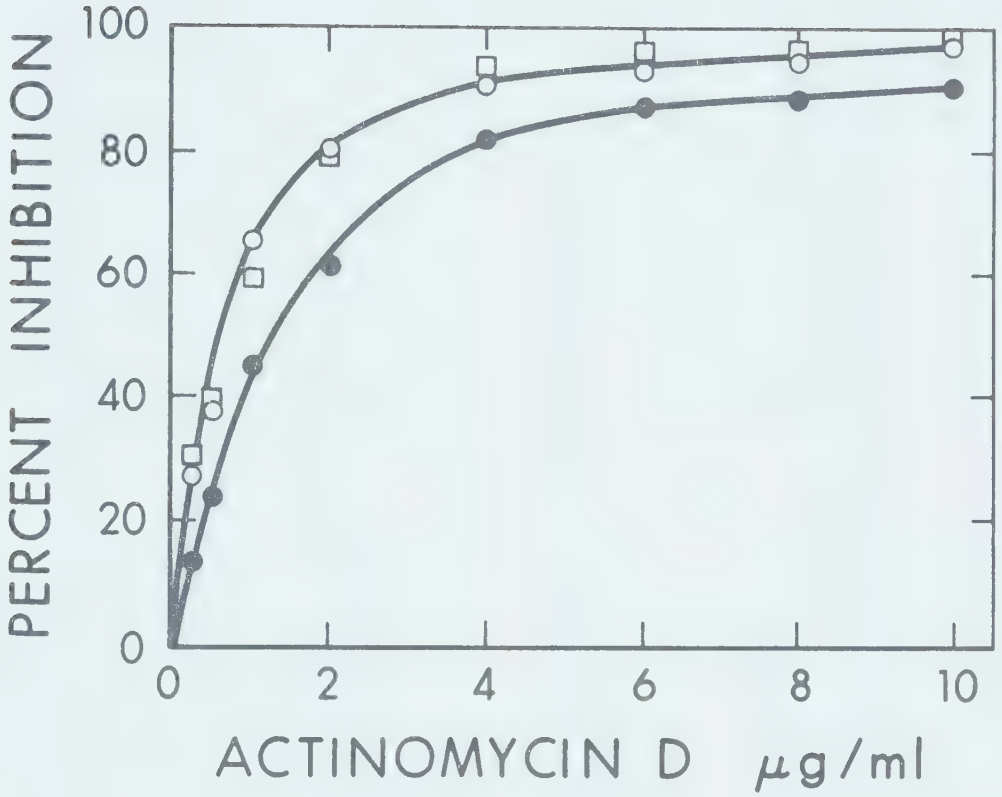

Figure 1. Effect of actinomycin D on conversion of radioactive purine bases to acid-insoluble nucleotides.

Incubation time: 60 min
(●) adenine-^{14}C, 100 μM
(□) guanine-^{14}C, 100 μM
(o) hypoxanthine-^{14}C, 100 μM

Table 1. Effect of Actinomycin D on enzymes of purine ribonucleotide synthesis and interconversion in Ehrlich ascites tumor cells *in vitro*. *

Parameter	Summation	Apparent Activities † (nmoles/g per 60 min) Control	Actinomycin D
Precursor: Adenine			
Adenine ⟶ ATP	ATP+NAD+RNA(A)	2865	2521
Adenine phosphoribosyltransferase	AMP+ADP+ATP+NAD+RNA(A)+IMP+XMP+GMP+GDP+GTP	3251	2693
AMP deaminase	IMP+GMP+GDP+GTP+XMP	203	50
ATP ⟶ Nucleic acid adenine	RNA(A)	178	22
Precursor: Guanine			
Guanine ⟶ GTP	GTP+RNA(G)	1493	926
Guanine phosphoribosyltransferase	GMP+GDP+GTP+RNA(G)+IMP+XMP+AMP+ADP+ATP+NAD	1649	1073
GMP reductase	IMP+XMP+AMP+ADP+ATP	85	95
GTP ⟶ Nucleic acid guanine	RNA(G)	309	21
Precursor: Hypoxanthine			
Hypoxanthine ⟶ ATP	ATP+NAD+RNA(A)	1651	991
Hypoxanthine phosphoribosyltransferase	IMP+AMP+ADP+ATP+NAD+RNA(A)+XMP+GMP+GDP+GTP+RNA(G)	2230	1280
Hypoxanthine ⟶ GTP	GTP+RNA(G)	243	62
Hypoxanthine phosphoribosyltransferase	IMP+AMP+ADP+ATP+NAD+RNA(A)+XMP+GMP+GDP+GTP+RNA(G)	2230	1280
IMP dehydrogenase	XMP+GMP+GDP+GTP+RNA(G)	300	106
ATP ⟶ Nucleic acid adenine	RNA(A)	89	8.2
GTP ⟶ Nucleic acid adenine	RNA(G)	107	2.7

* A 2% (v/v) suspension of Ehrlich ascites tumor cells was incubated with 100 µM purine base-^{14}C for 60 min in the presence and absence of 5 µg/ml actinomycin D.

† Apparent enzymatic activity for each parameter was calculated by the summation of radioactivity in the indicated compounds.

by 9% and radioactivity in guanine nucleotides synthesized from guanine-^{14}C by 24%. In view of these findings, a systematic examination of the effects of actinomycin D on acid-soluble purine metabolism was undertaken. The effect of actinomycin D on radioactive purine metabolism in Ehrlich ascites tumor cells is shown in Table 1.

The incorporation of adenine into nucleic acids was inhibited 88%, the actual inhibition of nucleic acid synthesis from ATP being 86%. An effect previously not distinguished from inhibition of nucleic acid synthesis was a 12% inhibition of ATP synthesis from adenine; this was the result of 17% inhibition of adenine phosphoribosyltransferase. The decrease in adenine nucleotide synthesis was somewhat compensated by a 70% reduction in the apparent activity of adenylate deaminase.

The effect of actinomycin D on the metabolism of guanine-^{14}C is also given in Table 1. Incorporation of guanine into nucleic acids was inhibited 93%, primarily due to 89% inhibition of GTP conversion to nucleic acid. The conversion of guanine to GTP was inhibited 38% and this is largely due to inhibition of guanine phosphoribosyltransferase and to a lesser extent, inhibition of guanine nucleotide kinases. The apparent activity of guanylate reductase was increased 72%.

The effects of actinomycin D on hypoxanthine metabolism (Table 1) are more complex than those on adenine or guanine, since appreciable amounts of hypoxanthine are converted to both ATP and GTP. The incorporation of hypoxanthine into nucleic acid adenine was inhibited 89%, the conversion of ATP to nucleic acid adenine being inhibited 84%. Hypoxanthine incorporation into nucleic acid guanine was inhibited 96%, GTP conversion to nucleic acid guanine being inhibited 84%.

Of particular interest are the effects of actinomycin D on the synthesis of ATP and GTP from hypoxanthine. The conversion of hypoxanthine to ATP was inhibited 33%, due to inhibition of hypoxanthine phosphoribosyltransferase, other effects being of a lesser degree. The conversion of hypoxanthine to GTP was inhibited 78%, primarily due to 33% inhibition of hypoxanthine phosphoribosyltransferase and 60% inhibition of IMP dehydrogenase. Previous observations (3,4) that incorporation of formate or hypoxanthine into RNA guanine was inhibited to a greater extent than that into RNA adenine are compatible with the inhibition of IMP dehydrogenase described here. The accumulation of IMP observed in B. subtilis exposed to actinomycin D would also indicate an effect on enzymes utilizing IMP (5).

The effect of a wide range of actinomycin D concentration on various aspects of hypoxanthine metabolism, are given in Figure 2. It is of interest that all of its inhibitory effects, including inhibition of nucleic acid synthesis (Fig. 1), have essentially the same dose response curve showing near maximal inhibition at 5 µg/ml actinomycin D.

One factor that several of the inhibited enzymes have in common is the substrate phosphoribosyl pyrophosphate (PRPP). Ehrlich ascites tumor cells incubated without glutamine for 60 min in the presence of 5 µg/ml actinomycin D showed a 40% reduction in the concentration of PRPP, and cells incubated with actinomycin D and glutamine showed a 29% reduction in the concentration of PRPP. Actinomycin D was shown to have no effect on the assay system for measuring PRPP concentration, which consisted of a crude preparation of adenine phosphoribosyltransferase from Ehrlich ascites tumor cells. Reduced concentrations of PRPP would also suggest that purine synthesis de novo should also be inhibited by actinomycin D. Purine synthesis de novo was measured by incorporation of glycine-^{14}C into phosphoribosylformylglycineamide; 5 µg/ml actinomycin D reduced this process by 11%. Thus limitation of this common substrate may be responsible for the observed

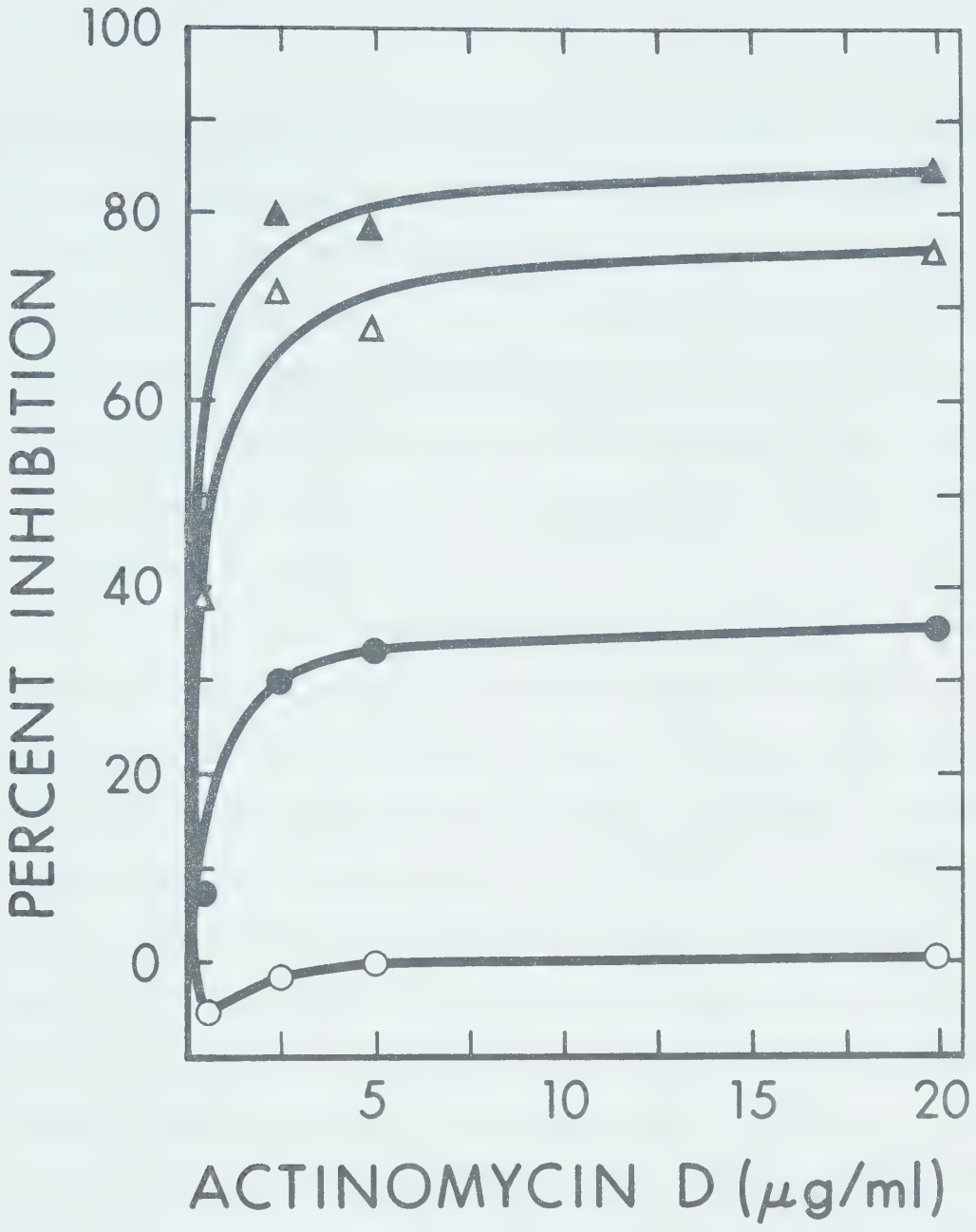

Figure 2. Effect of actinomycin D on the conversion of hypoxanthine and IMP to ATP and GTP.

Incubation time: 60 min
Precursor: hypoxanthine-^{14}C, 100 μM
(●) H → ATP, (▲) H → GTP
(○) IMP → ATP, (△) IMP → GTP

inhibition of four of the reactions measured. This possibility is further supported by the correlation between Km's for PRPP and the degree of inhibition by actinomycin D. These values are 5×10^{-6} M (15) and 17% inhibition for adenine phosphoribosyltransferase, and $2\text{-}4 \times 10^{-5}$ M (16) and 33% inhibition for hypoxanthine phosphoribosyltransferase with hypoxanthine as substrate and 35% inhibition with guanine as substrate.

There are several possible mechanisms whereby actinomycin D affects acid-soluble purine metabolism. Direct inhibition of enzyme activity appears unlikely for such a large molecule bearing little structural resemblance to metabolites, and actinomycin D was shown to have no direct effect on adenine phosphoribosyltransferase. It is known, however, that purine mononucleotides may interact with actinomycin D (3,17,18), but the extent and effect of such interactions in the whole cell is unknown.

Another possibility is that the observed inhibitions are secondary effects of inhibition of RNA synthesis. In addition, actinomycin D treatment has previously been shown to lead to a net breakdown of RNA (19,20). Liquid chromatography of cell extracts enabled a comparison to be made of nucleotide concentrations in cells incubated for 2 hours in the presence and absence of 2.5 µg/ml actinomycin D. Comparison of nucleotide pools before and

after the incubation period showed no significant change in the concentrations of nucleoside mono- and diphosphates; the concentration of GTP was reduced 0.8 fold, and that of ATP increased 1.2 fold. Following incubation with actinomycin D, the concentration of GTP was elevated 2.8 fold, and that of ATP, 1.3 fold.

One method of examining the possibility that secondary effects of inhibition of nucleic acid synthesis are involved, is to examine the effects of another inhibitor of nucleic acid synthesis on acid-soluble purine metabolism. Daunomycin has been shown to inhibit the conversion of labelled precursors to DNA and RNA in experimental tumors (2,21,22) and in Ehrlich ascites tumor cells the incorporation of hypoxanthine-^{14}C into nucleic acids was inhibited more than 90% by 50 µg/ml daunomycin. This concentration of daunomycin also inhibited the conversion of hypoxanthine to nucleic acid adenine by 98% and conversion to nucleic acid guanine by 99%. The conversion of hypoxanthine to ATP was inhibited 47%, primarily due to 45% inhibition of hypoxanthine phosphoribosyltransferase. The conversion of hypoxanthine to GTP was inhibited 83%, due to 45% inhibition of hypoxanthine phosphoribosyltransferase and 55% inhibition of IMP dehydrogenase. A comparison of the effects of daunomycin (50 µg/ml) and actinomycin D (5 µg/ml) on hypoxanthine metabolism shows

striking similarities. These results have suggested that it may be the secondary effects of nucleic acid synthesis inhibitors on acid-soluble nucleotide concentrations which are responsible for the observed effects on purine nucleotide metabolism. Further investigation of this possibility has been pursued (Chapter 7).

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CHAPTER 7

EFFECTS OF ELEVATED INTRACELLULAR ATP AND GTP CONCENTRATIONS ON PURINE RIBONUCLEOTIDE SYNTHESIS AND INTERCONVERSION

INTRODUCTION

Potential mechanisms for the regulation of the enzymes of purine ribonucleotide synthesis and interconversion have been studied in various cell-free systems, and the numerous observations of inhibition and stimulation of these enzymes by purine nucleotides have been reviewed (1,2). In contrast, the regulation of purine ribonucleotide synthesis and interconversion in intact animal cells has received relatively little attention.

That purine ribonucleotide synthesis is regulated is indicated by the observation that purine ribonucleotide concentrations in cultured animal cells are similar whether or not the cells are growing (3,4), that rates of conversion of purines to nucleotides is considerably less than the potential purine phosphoribosyltransferase activity, (5,6,7, Chapter 2), and that purine nucleotide synthesis from bases is not linear with time even at saturating concentrations of purines (6, Chapter 2). The mechanisms by which purine phosphoribosyltransferase activity in intact cells is regulated has not been defined, although in some cases PRPP concentrations are believed to be limiting.

Likewise, although Crabtree and Henderson (8,9) have identified the rate limiting reactions in the interconversion of purine ribonucleotides in Ehrlich ascites

tumor cells *in vitro*, the regulation of these reactions has so far been described only in terms of substrate concentration.

The present study examines the effects of increased purine ribonucleotide concentrations on the enzymes of purine nucleotide interconversion and synthesis from bases in a medium containing amino acids, so that interconversions were not limited by these substrates, and containing high concentrations of phosphate, which have been shown to promote nucleotide synthesis from purine bases (10).

MATERIALS AND METHODS

Sources of materials (Chapter 2), methods of tumor cell preparation (Chapter 2), procedures for the separation and measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides (8), have been reported previously. Coformycin was generously provided by Professor H. Umezawa and Dr. M. Hori, Institute of Microbial Chemistry, Tokyo.

A method for evaluating the effects of drugs or other factors on individual reactions of purine nucleotide metabolism in Ehrlich ascites tumor cells incubated with

hypoxanthine-^{14}C has been reported (11). Here it is extended to reactions measured when cells were incubated with radioactive adenine or guanine. Results are presented in terms of the amount of radioactive substrate metabolized by the different enzymes in a given period; other studies (8) have indicated that under these conditions radioisotope flow from purine bases to ATP and GTP is virtually equivalent to the observed changes in chemical composition of the triphosphate pools.

RESULTS

Analysis of the concentrations of individual purine nucleotides in Ehrlich ascites tumor cells following incubation in the presence and absence of adenine or guanine (Table 1), shows that ATP and GTP concentrations can be significantly increased by this procedure. Only an upper limit could be assigned to the concentration of GDP and the monophosphates AMP, IMP and GMP were present in amounts less than 100 nmoles/g. Yushok (12) has shown by enzymatic analysis that 60 min incubation of Ehrlich ascites tumor cells in Krebs-Ringer phosphate with 1.0 mM adenine or adenosine increased ATP concentrations from 3630 to 7300 nmoles/g, those of ADP from 220 to 300 nmoles/g, and those of AMP

Table 1. Elevation of intracellular ATP and GTP concentrations.

Incubation with Adenine* (μM)	Ribonucleotide (nmoles/g)						Relative change in ribonucleoside triphosphate concentration	
	AMP	ADP	ATP	GMP	GDP	GTP	$ATP^{exp}/ATP^{control}$	$GTP^{exp}/GTP^{control}$
0	-†	175	3020	-	-	860	1.0	1.0
100	-	290	3595	-	-	800	1.20	0.95
Incubation with Guanine* (μM)								
0	-	210	3010	-	<100	1210	1.0	1.0
20	-	230	3200	-	<100	1705	1.05	1.40
100	-	245	3765	-	<100	3010	1.25	2.50
200	-	280	3770	-	<100	4580	1.25	3.80

* Ehrlich ascites tumor cells, 2% (v/v) suspension, were incubated with unlabelled adenine or guanine for 30 min.

† Due to their low concentration, only an upper limit of 100 nmoles/g could be assigned to AMP, GMP, and GDP.

from 50 to 160 nmoles/g. Since the total increase in triphosphate pools is much greater than changes at the mono- and diphosphate level, changes in enzyme activities have been correlated with increases in either ATP or GTP intracellular concentrations.

Ehrlich ascites tumor cells were preincubated with adenine (100 μM) for 30 min, which increased the ATP concentration 1.2 fold, whereas the GTP concentration was 0.95 that of control cells (Table 1). Control cells and cells with elevated ATP concentrations were then washed, resuspended in fresh prewarmed medium and incubated with 200 μM adenine-^{14}C, hypoxanthine-^{14}C, or guanine-^{14}C for 60 min, and the apparent activities of several enzymes of purine nucleotide metabolism were calculated. The data in Table 2 shows that the conversion of adenine, hypoxanthine and guanine to nucleotide was inhibited between 45 and 52% in cells with elevated ATP concentrations.

Reduced phosphoribosyltransferase activity may have been the result of a reduction in the concentration of PRPP induced by the prior incubation with unlabelled adenine. In order to examine this possibility, the ATP pool was elevated by incubation with adenosine in the presence of coformycin (1 μg/ml); the latter has been shown to inhibit adenosine deaminase by greater than 99% in these

Table 2. Effects of elevated intracellular ATP concentrations on enzyme activities*

Enzyme	Inhibition of Enzyme Activity (%)
Precursor: Adenine	
Adenine phosphoribosyltransferase (3280†)	44.8
AMP kinase (3200†)	1.3
Precursor: Guanine	
Guanine phosphoribosyltransferase (1765†)	51.8
GMP kinase (1625†)	6.9
Precursor: Hypoxanthine	
Hypoxanthine phosphoribosyltransferase (2630†)	47.6
AMPS synthetase plus lyase (2140†)	8.9

* Control Ehrlich ascites tumor cells, 2% (v/v) suspension, and cells with elevated ATP concentration ($ATP^{experimental}/ATP^{control} = 1.20$) were incubated with 200 μM adenine-^{14}C, guanine-^{14}C, or hypoxanthine-^{14}C for 60 min.

† Apparent activity for control cells, nmoles/g per 60 min.

cells (13), and hence adenosine could not be converted to hypoxanthine, which also would react with PRPP. Incubation of Ehrlich ascites tumor cells with adenine (50 μM), or adenosine (50 μM) plus coformycin (1 μg/ml) gave comparable increases in the concentrations of ATP, 1.56 and 1.52 fold, respectively. Figure 1 shows that nucleotide synthesis from adenine-^{14}C was inhibited in cells containing elevated ATP concentrations whether they had been incubated with either adenine or adenosine.

Ehrlich ascites tumor cells were also preincubated with three concentrations of guanine for 30 min to elevate the concentrations of GTP, as shown in Table 1. Following the incubation with non-radioactive guanine, cells were washed and resuspended in fresh medium and incubated with radioactive purine bases for 60 min and radioactivity in nucleotides measured. The relative activity of various enzymes were plotted versus the relative increase in the GTP concentration (Fig. 2).

It may be seen from Figure 2 that increasing GTP concentrations caused inhibition of nucleotide synthesis from radioactive adenine, guanine and hypoxanthine, the order of inhibition corresponding to that caused by elevated ATP levels. The relative activities of AMP kinase and GMP kinase were virtually unaffected by increasing GTP concentrations and the relative activity of adenylosuccinate

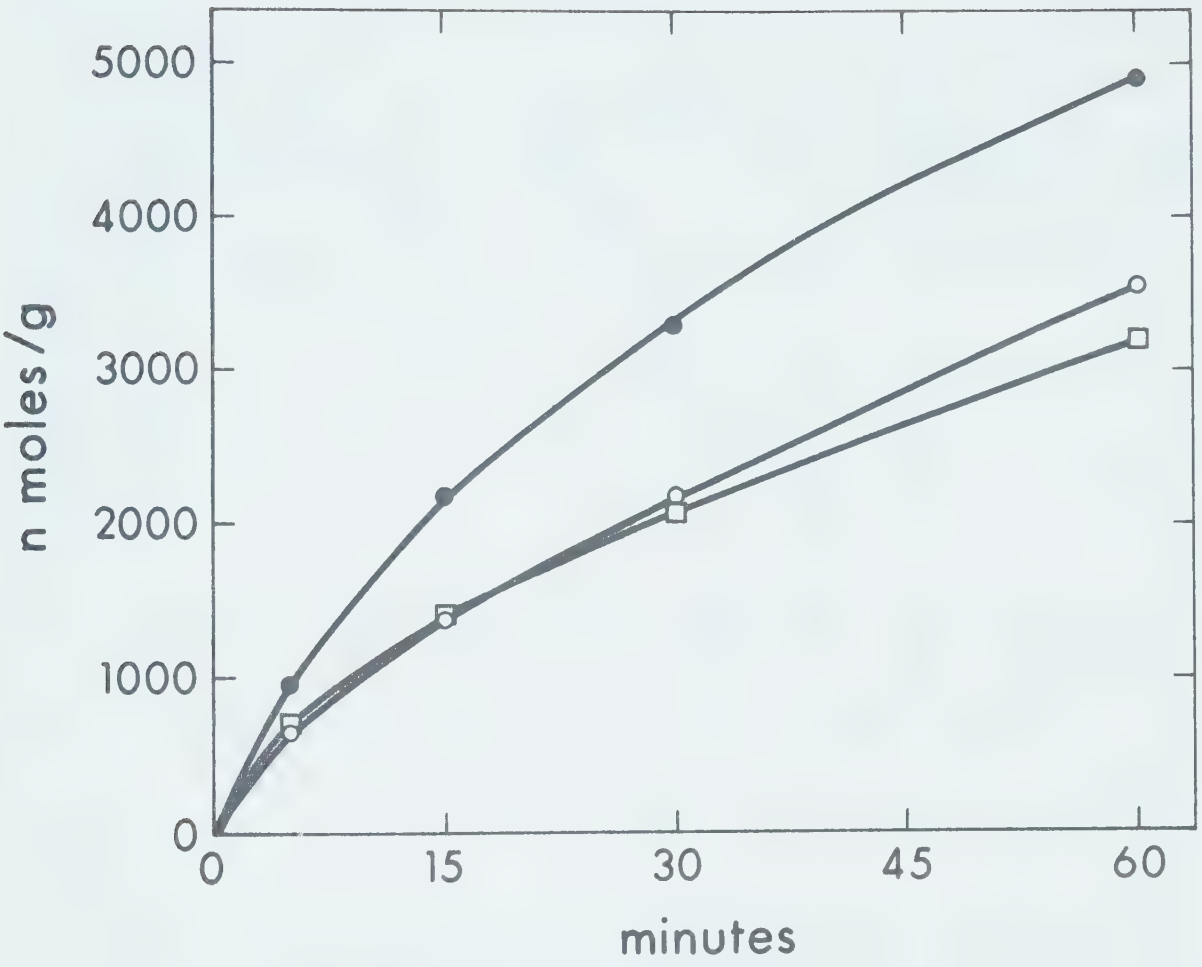

Figure 1. Effect of elevated ATP concentrations on acid-soluble nucleotide synthesis from adenine-^{14}C.

(●) control cells
(□) cells incubated with adenine
$ATP^{exp} / ATP^{control} = 1.56$
$GTP^{exp} / GTP^{control} = 0.95$
(o) cells incubated with adenosine plus coformycin
$ATP^{exp} / ATP^{control} = 1.52$
$GTP^{exp} / GTP^{control} = 1.01$
Precursor: adenine-^{14}C, 200 μM

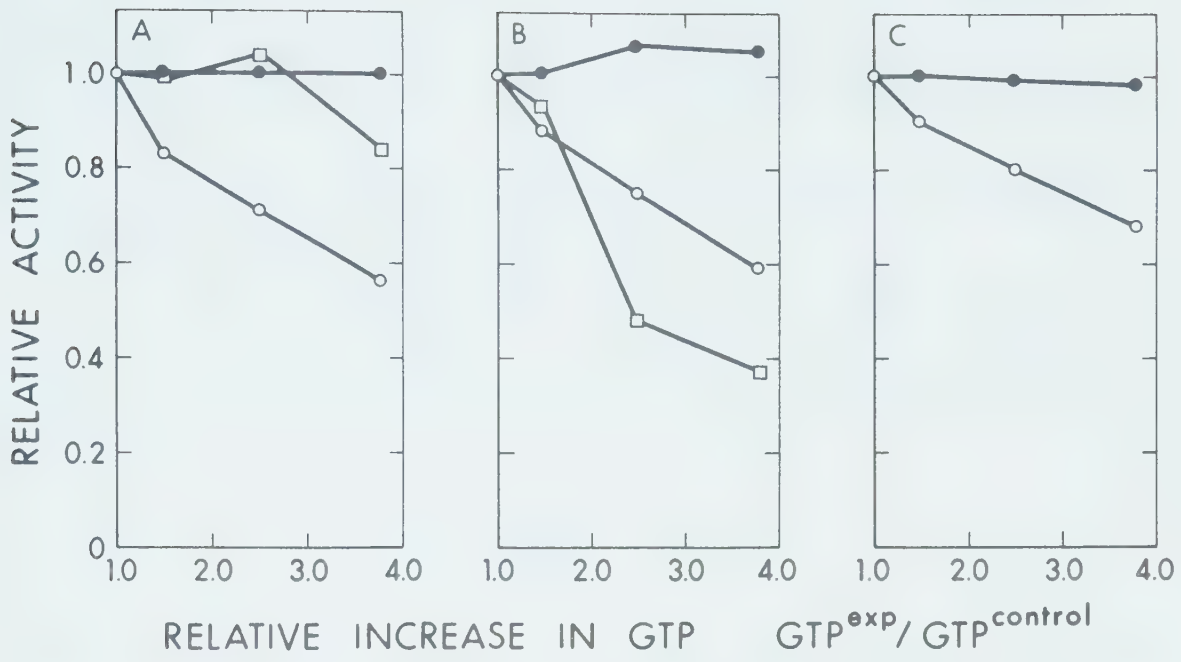

Figure 2. Effect of elevated GTP concentrations on enzymes of purine ribonucleotide synthesis and interconversion.

Precursor: A; adenine-^{14}C, 200 μM
B; hypoxanthine-^{14}C, 200 μM
C; guanine-^{14}C, 200 μM

Enzymes:
A, (o) adenine phosphoribosyltransferase; control = 4180*
(●) AMP kinase; control = 4085*
(□) AMP deaminase; control = 90*
B, (o) hypoxanthine phosphoribosyltransferase; control = 3290*
(●) adenylosuccinate synthetase plus lyase; control = 2643*
(□) IMP dehydrogenase; control = 355*
C, (o) guanine phosphoribosyltransferase; control = 2310*
(●) GMP kinase; control = 2110*

* nmoles/g per 60 min

synthetase plus lyase was slightly increased. AMP deaminase activity was inhibited only at the highest concentration of GTP, but the relative activity remained high (0.84) under these conditions. The most dramatic effect was a decrease in the relative activity of IMP dehydrogenase to 0.37 of that for the control cells.

Neither ADP kinase, GDP kinase or GMP synthetase were affected by increases in ATP or GTP concentration.

DISCUSSION

These experiments have for the first time demonstrated inhibition of certain reactions of purine metabolism by ATP and GTP in intact animal cells. Purine phosphoribosyltransferase activities were inhibited at high ATP and GTP concentrations, but whether inhibition is due to a direct effect on these enzymes, or due to inhibition of PRPP synthesis, has not been distinguished. The adenine phosphoribosyltransferase of Ehrlich ascites tumor cells is inhibited competitively with respect to PRPP by AMP, ADP, and ATP (14-19), and the enzyme is also inhibited by dAMP and GMP by different mechanisms (18,20). Hypoxanthine-guanine phosphoribosyltransferase of Ehrlich ascites tumor cells is also competitively inhibited by GMP and IMP with respect to PRPP (14). Increases

in the concentrations of AMP, IMP and GMP were not detectable, however, and the inhibition of both enzymes by elevated ATP or GTP concentrations suggests a common element.

It is also possible that inhibition of PRPP synthetase by elevated nucleotide concentrations is the cause of the observed effects. Wong and Murray (21) have shown that PRPP synthetase of Ehrlich ascites tumor cells is partially inhibited by AMP, GMP and IMP, and also by several nucleoside triphosphates; half-maximal inhibitions were obtained with 0.25 mM CTP, 0.2 mM GTP, 0.4 mM TTP and 0.4 mM UTP. Inhibition of phosphoribosyltransferases may therefore be a composite of both product inhibition and decreased PRPP synthesis.

Adenylate and guanylate kinases were virtually unaffected by elevated ATP and GTP concentrations. Higher energy charge in these cells might be expected to support further phosphorylation of mononucleotides and this seemed to be the case.

Adenylosuccinate synthetase and adenylosuccinate lyase activities were examined as a single coordinate system that showed a small degree of inhibition (9%) at high concentrations of ATP and slightly increased relative activity (1.05) in cells having elevated GTP concentrations. Little is known about these enzymes from mammalian sources, however,

certain purine nucleotides, AMP, GMP and GDP inhibit the adenylosuccinate synthetase of Escherichia coli (22,23), and AMP, though not ATP, inhibits adenylosuccinate lyase of yeast (24). The conversion of IMP to AMP in Ehrlich ascites tumor cells was affected only to a minor degree by increased ATP and GTP concentration.

In Ehrlich ascites tumor cells, adenylate deaminase activity relative to AMP kinase activity is very low as reflected by the ratio of AMP formed from adenine which was further phosphorylated, 4085 nmoles/g per hr, to that which was deaminated, 90 nmoles/g per hr. Thus adenylate deaminase activity may normally be strictly regulated in these cells as indicated in the work of Lomax and Henderson (personal communication). Only cells with the highest relative increase of GTP showed a partial inhibition of this enzyme. Studies of adenylate deaminase from a variety of sources have showed that this enzyme is stimulated by ATP and inhibited by GTP (25,26,27). Inhibition by GTP as observed in these studies, although in accord with other findings, suggest that this enzyme is not as sensitive to inhibition by GTP in intact Ehrlich ascites tumor cells as might have been expected. It should be noted that adenylate deaminase of skeletal muscle from several sources was inhibited by inorganic phosphate and ATP, the latter perhaps only at low concentrations (28,29), and ADP was also

suggested to be an activator of this enzyme (28).

IMP dehydrogenase activity in Ehrlich ascites tumor cells was found to be strongly inhibited by increases in GTP concentrations (Fig. 2B), although studies of partially purified IMP dehydrogenases from Ehrlich ascites tumor cells (30) and Sarcoma 180 cells (31) gave no indication of allosteric properties. The mammalian enzyme is inhibited by XMP and reduced nicotinamide adenine dinucleotide (31). In detailed studies of this enzyme from microorganisms, GMP has generally been regarded as a feedback inhibitor for regulation of guanine nucleotide synthesis (32,33). At present, a study of the effect of guanine nucleotides on cell-free IMP dehydrogenase from mammalian sources has not been conducted.

In summary, high ATP and GTP concentrations inhibit nucleotide overproduction from purine bases via the phosphoribosyltransferases. Elevated concentrations of ATP may have a slight inhibitory effect on AMP synthesis from IMP, whereas high GTP concentrations severely inhibit IMP dehydrogenase. At concentrations of GTP greater than ATP, a moderate inhibition of adenylate deaminase was observed. In general, the activities of enzymes of purine nucleotide interconversion appear to be more strictly subject to increased GTP concentrations than to ATP concentrations.

These observations would also support the

hypothesis that the effects of actinomycin D and daunomycin on acid-soluble purine metabolism (Chapter 6) may be due to increases in the intracellular ribonucleotide concentrations, especially GTP.

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CHAPTER 8

ALTERNATIVE PATHWAYS OF DEOXYADENOSINE AND ADENOSINE METABOLISM

INTRODUCTION

Deoxyadenosine and adenosine can be metabolized in animal cells both by phosphorylation and by deamination. The relative rates of phosphorylation and deamination of deoxyadenosine are known to vary from one animal cell type to another, but their relationship has not been studied systematically. In Ehrlich ascites tumor cells incubated with deoxyadenosine, for example, large amounts of dADP and dATP were found to accumulate (1,2). Withdrawal of the deoxyadenosine, however, resulted in rapid degradation of dATP primarily to hypoxanthine, although small amounts of deoxyadenosine and deoxyinosine were also observed (1). In contrast, radioactivity from deoxyadenosine was incorporated primarily into RNA rather than into DNA in human lymphocytes (3), suggesting that deamination followed by cleavage of the glycosidic bond of deoxyinosine was of major importance.

Recently Zimmerman et al. (4) have shown that purine nucleoside phosphorylase can convert adenine to adenosine, and this observation raises the possibility that adenine nucleosides can also be cleaved to adenine in intact animal cells. This possibility is explored in the present study, which also determines the relative rates of deoxyadenosine metabolism via phosphorylation, deamination

and cleavage in several different animal cells and tissues.

METHODS AND MATERIALS

Chemicals

Radioactive bases and nucleosides were purchased from New England Nuclear Corporation: deoxyadenosine-8-^{14}C, 39 mCi/mmole; inosine-8-^{14}C, 32 mCi/mmole; adenine-8-^{14}C, 52.6 mCi/mmole; guanine-8-^{14}C, 52 mCi/mmole; and from Schwarz BioResearch Inc.: adenosine-8-^{14}C, 47 mCi/mmole; hypoxanthine-8-^{14}C, 53.7 mCi/mmole. Coformycin was generously provided by Professor H. Umezawa and Dr. M. Hori, Institute of Microbial Chemistry, Tokyo.

Deoxyadenosine-^{14}C was purified prior to each experiment by chromatographing 100 to 200 µl on Eastman Kodak unsubstituted cellulose thin layer sheets in butanol-methanol-water-ammonia (60:20:20:1); sheets were developed twice. This procedure separated deoxyadenosine from adenine, adenosine, hypoxanthine and inosine. The deoxyadenosine spot was scraped off the chromatogram and extracted with four 0.5 ml aliquots of water. The combined aqueous extracts were evaporated to dryness in vacuo over NaOH flakes and resuspended in the original sample volume. Only deoxyadenosine preparations free from radioactive adenine,

adenosine, inosine and hypoxanthine were used for the experiments reported.

Adenosine deaminase in Ehrlich ascites tumor cells has been shown to be inhibited by more than 99% by 1 µg/ml coformycin (5). In this study, 5 µg/ml coformycin was used in experiments employing mouse, sheep and human erythrocytes and Ehrlich ascites tumor cells, whereas 10 µg/ml was used when preparations of mouse brain, kidney, heart and lung were studied. Control experiments showed that in all cases the deamination of deoxyadenosine-^{14}C was virtually completely inhibited by these concentrations of coformycin.

Tissue preparation and incubation conditions

Ehrlich ascites tumor cells were collected and diluted in Fischer's medium containing 25 mM phosphate buffer, pH 7.4, without bicarbonate, to make a 2.5% cell suspension. The tumor cell suspensions (80 µl) with or without coformycin were incubated in small plastic tubes (10 x 75 mm, Falcon Plastics) for 20 min at 37° with shaking. Radioactive nucleoside and Fischer's medium was then added to a final volume of 100 µl, and the incubation continued. Experiments were terminated by addition of 5 µl of 4.2 M cold perchloric acid to each tube, and the cell extract was neutralized by the addition of 5 µl of 4.42 M KOH. Samples were chilled, centrifuged and 10 µl of the

supernatant was chromatographed.

Mouse, sheep and human erythrocytes were collected in modified Fischer's medium containing heparin, then washed and diluted to 2.5% suspension in modified Fischer's medium and incubated as above with radioactive precursors in the presence or absence of coformycin with 100% O_2 in the gas phase.

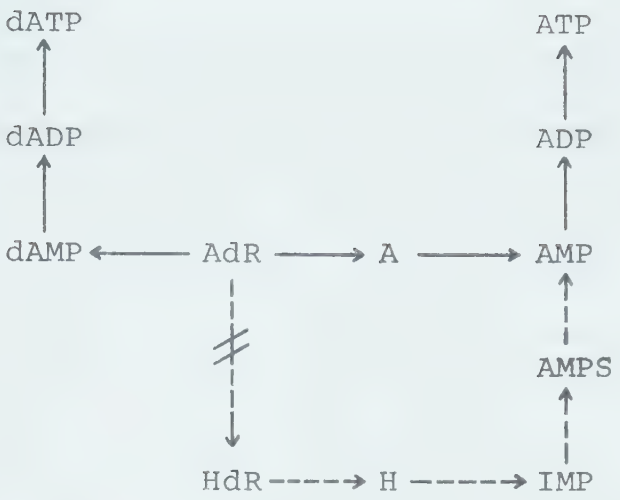
Deoxyadenosine-^{14}C metabolism was also examined in four mouse tissues. Adult Ha/ICR Swiss male mice were decapitated, organs removed and rinsed briefly in cold 0.154 M NaCl. Slices 2 mm thick were prepared from brain, liver and kidney. Slices were then chopped in two dimensions by a McIlwain tissue chopper (Brinkman Instruments Ltd., Toronto, Ontario, Canada), so that prisms 0.3 x 0.3 x 2 mm were obtained (6). Heart slices 1 mm thick were prepared by hand.

Portions of chopped or sliced tissue (5 to 20 mg) were transferred to tubes containing 0.200 ml modified Fischer's medium. Tissues were dispersed by vigorous shaking and incubated in a shaking water bath for 20 min at 37° with 100% O_2 in the gas phase. The medium was then removed by aspiration and the tissue was washed once with 0.200 ml prewarmed medium and then resuspended in 0.080 ml fresh medium with or without coformycin, and incubated for a further 15 min. Deoxyadenosine-^{14}C and Fischer's medium was

then added so the final volume after additions was 0.100 ml, and the incubation continued for a further 20 min. To terminate incubations, 5 µl of 4.2 M perchloric acid was added to each tube and the contents homogenized and then neutralized with 5 µl of 4.42 M KOH. The extracts were kept on ice for 5 min before centrifugation and 10 or 20 µl of the acid-soluble supernatant was chromatographed for 2 dimensional separation of ribo- and deoxyribonucleotides as described in Chapter 5.

RESULTS

In view of the demonstration by Zimmerman et al. (4) that adenine could be converted to adenosine by animal cell enzymes, the possibility that deoxyadenosine could be cleaved to adenine in intact animal cells was studied. This was first studied in the presence of coformycin, an inhibitor of adenosine deaminase, so that phosphorylation and cleavage of deoxyadenosine can be clearly distinguished in cells which perform both reactions simultaneously (Scheme 1).



SCHEME 1

Deoxyadenosine kinase activity will give rise to the radioactive deoxyadenosine phosphates dAMP, dADP and dATP, whereas the cleavage of deoxyadenosine will give rise to radioactive adenine; because of adenine phosphoribosyltransferase activity in the cells, the adenine so formed will be converted to the radioactive adenosine phosphates AMP, ADP and ATP. In the experiments described below only very small amounts of free radioactive adenine were detected; these were not attributed to deoxyadenosine cleaving activity because the perchloric acid extraction procedure itself caused a small and variable amount of hydrolysis of deoxyadenosine-^{14}C to adenine. The term "cleavage" is used in reference to the formation of adenine from deoxyadenosine because of difficulty encountered in distinguishing between phosphorolytic and hydrolytic processes in this

intact cell system.

Initial evidence for the cleavage of the glycosidic bond of deoxyadenosine in Ehrlich ascites tumor cells was obtained by such measurements of radioactivity in adenine ribo- and deoxyribonucleotides. Figure 1 shows the relative rates of deoxyadenosine kinase and deoxyadenosine cleaving activities in Ehrlich ascites tumor cells as a function of substrate concentration; both appeared near saturation at 56 μM deoxyadenosine. Deoxyadenosine kinase activity was twice that of the cleaving activity over the concentration range studied. These observations constitute the first firm evidence for adenine formation from a nucleoside in intact animal cells.

Deoxyadenosine kinase and cleaving activities were also measured in human, mouse and sheep erythrocytes. Human erythrocytes exhibited the highest activity of deoxyadenosine kinase and sheep erythrocytes the lowest (Fig. 2). Deoxyadenosine cleaving activities (Fig. 3) were three to six-fold lower than the corresponding kinase activities .

Chopped prisms of mouse kidney, liver and brain and slices of mouse heart were incubated with 20 μM deoxyadenosine-^{14}C in the presence of coformycin (10 μg/ml) for 20 min. Under these conditions there was no detectable deamination of deoxyadenosine, judged by the lack of accumulation of radioactivity in deoxyinosine and hypoxanthine.

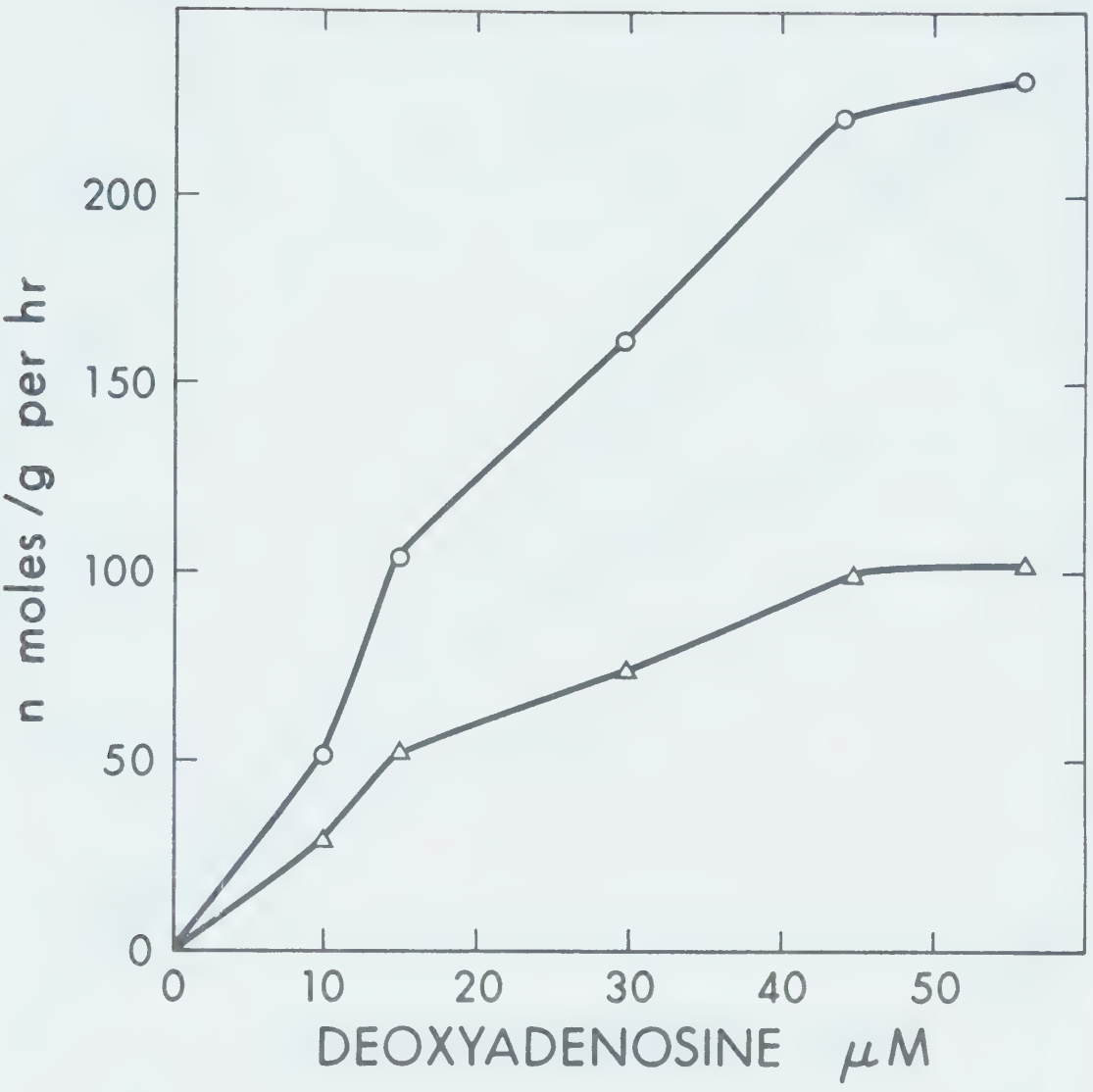


Figure 1. Metabolism of deoxyadenosine-^{14}C in Ehrlich ascites tumor cells.

Cells were incubated with deoxyadenosine-^{14}C for 20 min and radioactivity in the products of deoxyadenosine kinase, dAMP+dADP+dATP (o) and deoxyadenosine cleavage, AMP+ADP+ATP (△) was measured. The concentration of coformycin was 5 μg/ml.

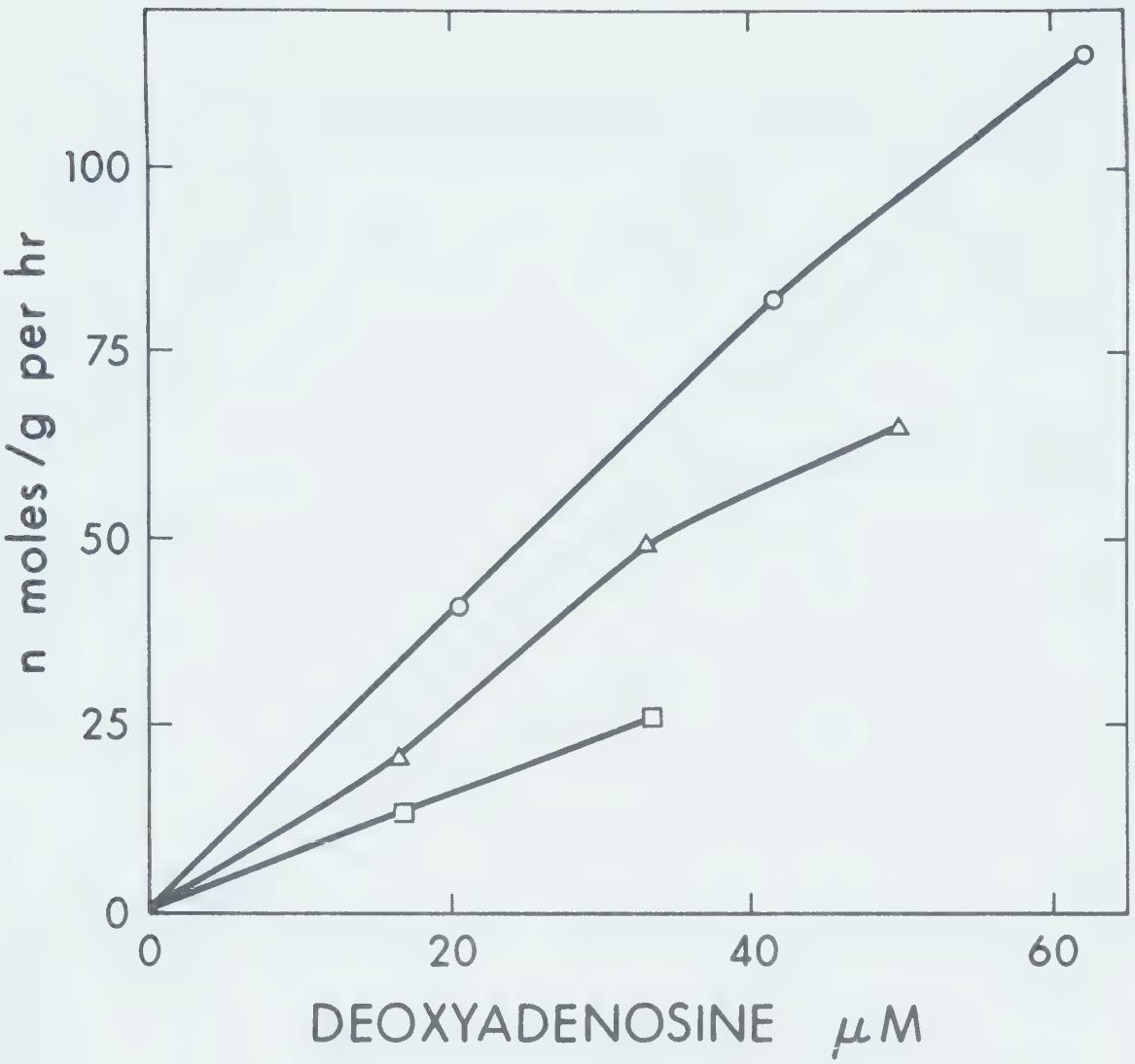


Figure 2. Phosphorylation of deoxyadenosine-^{14}C in erythrocytes.

Human (o), mouse (△), and sheep (□) erythrocytes were incubated with deoxyadenosine-^{14}C for 30 min after which radioactivity in dAMP+dADP+dATP was measured. Human and mouse erythrocytes were incubated with 5 μg/ml coformycin and sheep erythrocytes in the absence of coformycin.

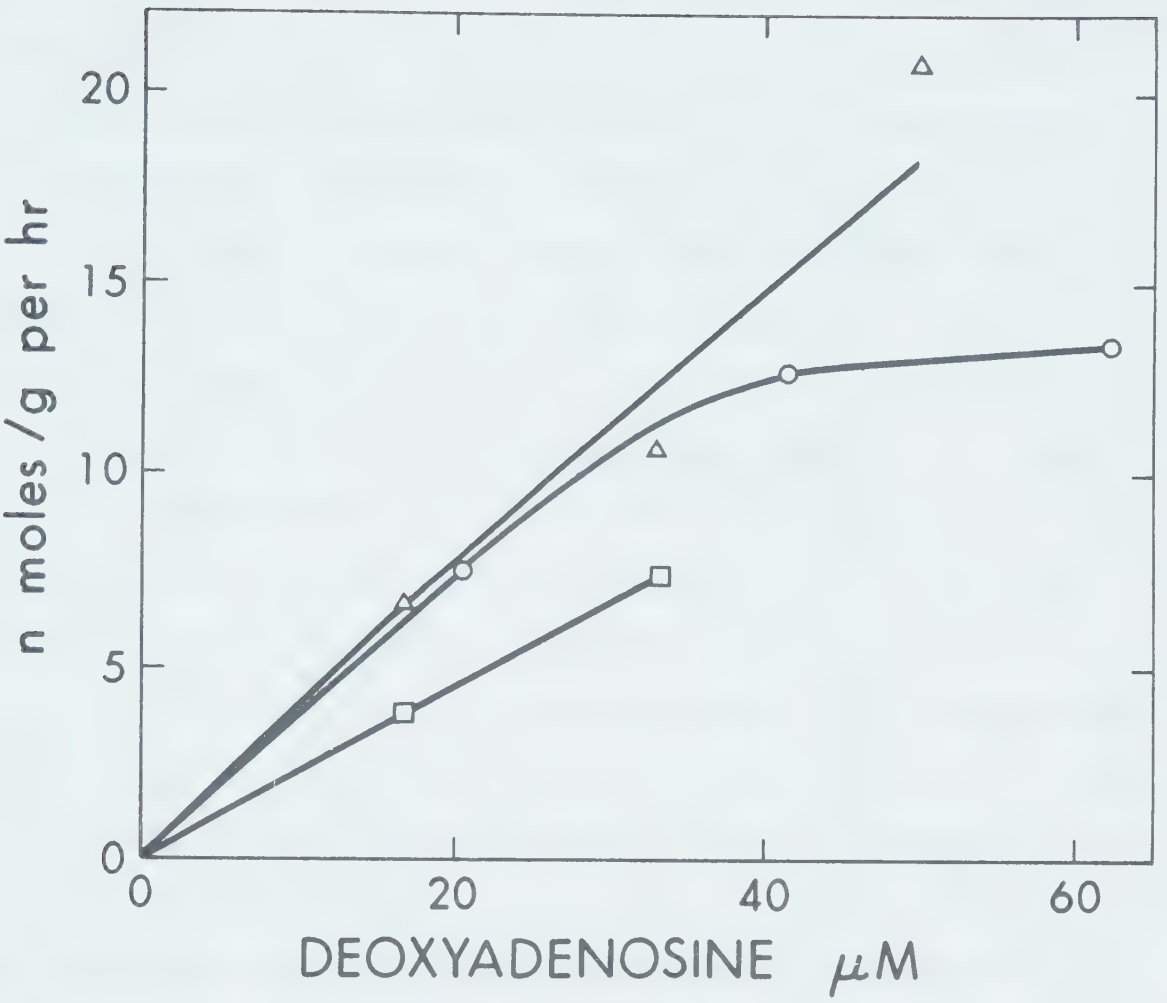


Figure 3. Cleavage of deoxyadenosine-^{14}C in erythrocytes.

Human (o), mouse (△), and sheep (□) erythrocytes were incubated with deoxyadenosine-^{14}C for 30 min after which radioactivity in AMP+ADP+ATP was measured. Human and mouse erythrocytes were incubated with 5 μg/ml coformycin and sheep erythrocytes in the absence of coformycin.

All of these tissues had sufficient adenine phosphoribosyltransferase under the conditions of these experiments for the assay of deoxyadenosine cleavage (7; P. C. L. Wong and J. F. Henderson, unpublished results). Figure 4 shows that of the four mouse tissues studied, deoxyadenosine kinase and cleaving activities were highest in kidney; brain and liver had comparable activities which were lower then kidney, and these activities in heart were close to the lower limit of sensitivity of the methods used. A comparison of deoxyadenosine kinase and cleaving activities in all the cells and tissues examined is given in Table 1.

The metabolism of deoxyadenosine was also measured in the absence of coformycin to determine the relative rates of phosphorylation and deamination of this nucleoside. Incubation of mouse liver, kidney, heart or brain for 20 min in the absence of coformycin resulted in the deamination of more than 90% of the 20 μM deoxyadenosine-^{14}C present as measured by radioactivity in deoxyinosine and hypoxanthine; ribonucleotides accounted for a further 1-5% of the total radioactivity. In these tissues cleavage cannot be measured in the presence of such extensive deaminase activity because deoxyinosine can be converted to adenine ribonucleotides via hypoxanthine and inosinate.

In contrast, there was essentially no synthesis of ATP or GTP from radioactive hypoxanthine in both human

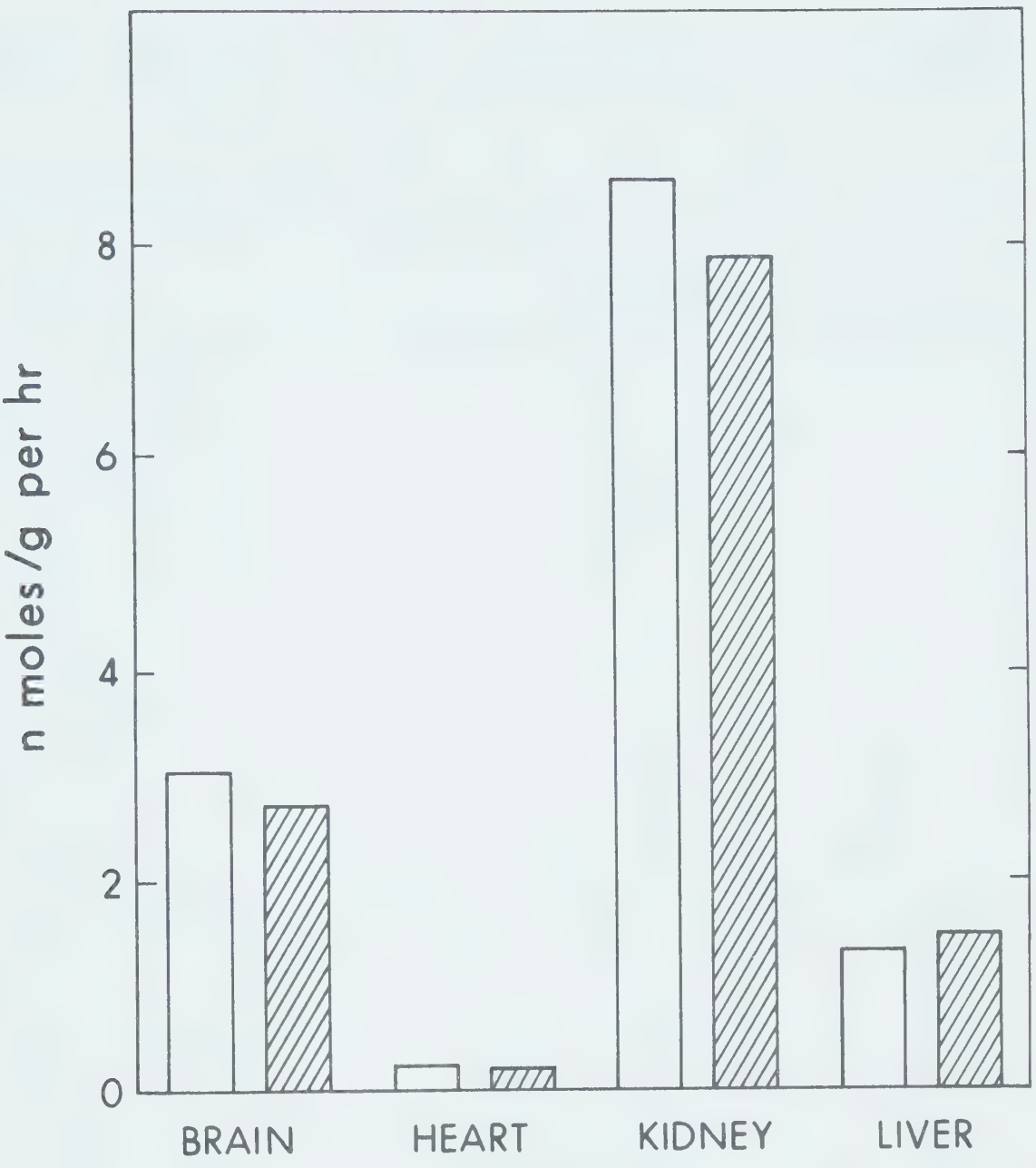


Figure 4. Metabolism of deoxyadenosine-^{14}C in mouse tissue.

Tissues were incubated with deoxyadenosine (20 µM) for 20 min and radioactivity in the products of deoxyadenosine kinase, dAMP+dADP+dATP (open bar) and deoxyadenosine cleavage, AMP+ADP+ATP (hatched bar) was measured.
The concentration of coformycin was 10 µg/ml.

Table 1. Relative activities of deoxyadenosine phosphorylation and cleavage.

Tissue	Coformycin (μg/ml)	Deoxyadenosine Concentration (μg/ml)	Phosphorylation / Cleavage
Human erythrocytes	5	20 - 42	6.0
Mouse erythrocytes	5	16 - 50	3.6
Sheep erythrocytes	0	16 - 33	3.5
Ehrlich ascites tumor cells	5	10 - 56	2.1
Mouse brain	10	20	1.2
Mouse kidney	10	20	1.1
Mouse heart	10	20	1.0
Mouse liver	10	20	0.9

and sheep erythrocytes, and deoxyadenosine cleaving activity can hence be measured in the absence of coformycin. Deoxyadenosine deaminase, kinase and cleaving activities in human and sheep erythrocytes incubated without coformycin are given in Table 2. In human erythrocytes, deamination of deoxyadenosine was some 75 to 180 fold greater than phosphorylation, whereas in sheep erythrocytes deamination and phosphorylation of deoxyadenosine were roughly equivalent. The ratio of cleaving activity to those of the other processes was several fold higher in sheep erythrocytes than in human erythrocytes, although total cleaving activity was higher in human erythrocytes.

Alternative pathways of adenosine metabolism in Ehrlich ascites tumor cells and sheep erythrocytes were also examined. Miech and Santos (8), by the use of adenosine labelled both in the base and sugar moieties, have previously concluded that in rat erythrocytes phosphorylation was the major route of adenine nucleotide synthesis from adenosine. In the present study, adenosine kinase and cleaving activities cannot be distinguished by the methods used, since AMP may be formed either by phosphorylation, or following cleavage or deamination of adenosine. However, the sum of radioactivity in hypoxanthine and inosine following incubation with adenosine-^{14}C has been taken to be a measure of adenosine deaminase activity and

Table 2. Comparison of deoxyadenosine deaminase, kinase and cleavage activities in human and sheep erythrocytes*

Deoxyadenosine (μM)	Deaminase	Kinase	Cleavage	Relative Activities†		
	(nmoles/g per hr)			Deaminase	Kinase	Cleavage
HUMAN ERYTHROCYTES						
20.7	1865	10.4	7.9	99.0	0.6	0.4
41.6	3484	36.1	13.4	98.6	1.0	0.4
62.0	3619	49.3	20.4	98.1	1.3	0.6
SHEEP ERYTHROCYTES						
16.7	14.8	13.2	3.8	46.5	41.5	12.0
33.3	24.3	26.1	7.4	42.0	45.2	12.8

* A 2% (v/v) suspension of erythrocytes were incubated with deoxyadenosine-^{14}C for 20 min with human erythrocytes or 60 min with sheep erythrocytes.

† Relative activities, where deaminase + kinase + cleavage = 100.0.

radioactivity in AMP, ADP and ATP to be a measure of adenosine kinase activity.

A comparison of adenosine kinase and deaminase activities in intact Ehrlich ascites tumor cells is shown in Figure 5. At low substrate concentrations several times more adenosine is phosphorylated than deaminated, as would be predicted from the Michaelis constants of adenosine for adenosine kinase, 3-6 x 10^{-6} M (9,10), and adenosine deaminase, 3 x 10^{-5} M (11). At high concentrations of adenosine (greater than 150 μM), deamination of adenosine was greater than phosphorylation. Phosphorylation of adenosine by Ehrlich ascites tumor cells was 5-fold greater than that of deoxyadenosine at concentrations of 16 μM.

In sheep erythrocytes adenosine deaminase activity is shown in Figure 6 to be greater than adenosine kinase activity over the whole range of adenosine concentrations studied. Deamination of deoxyadenosine was 4-fold lower than deamination of equal concentrations of adenosine in these cells. Adenosine and deoxyadenosine kinase activities were nearly equivalent, but were low in comparison to other cells. To determine whether the low activities of enzymes of adenosine and deoxyadenosine metabolism in sheep erythrocytes were related to limited uptake of nucleoside, these cells were incubated with inosine-^{14}C, and

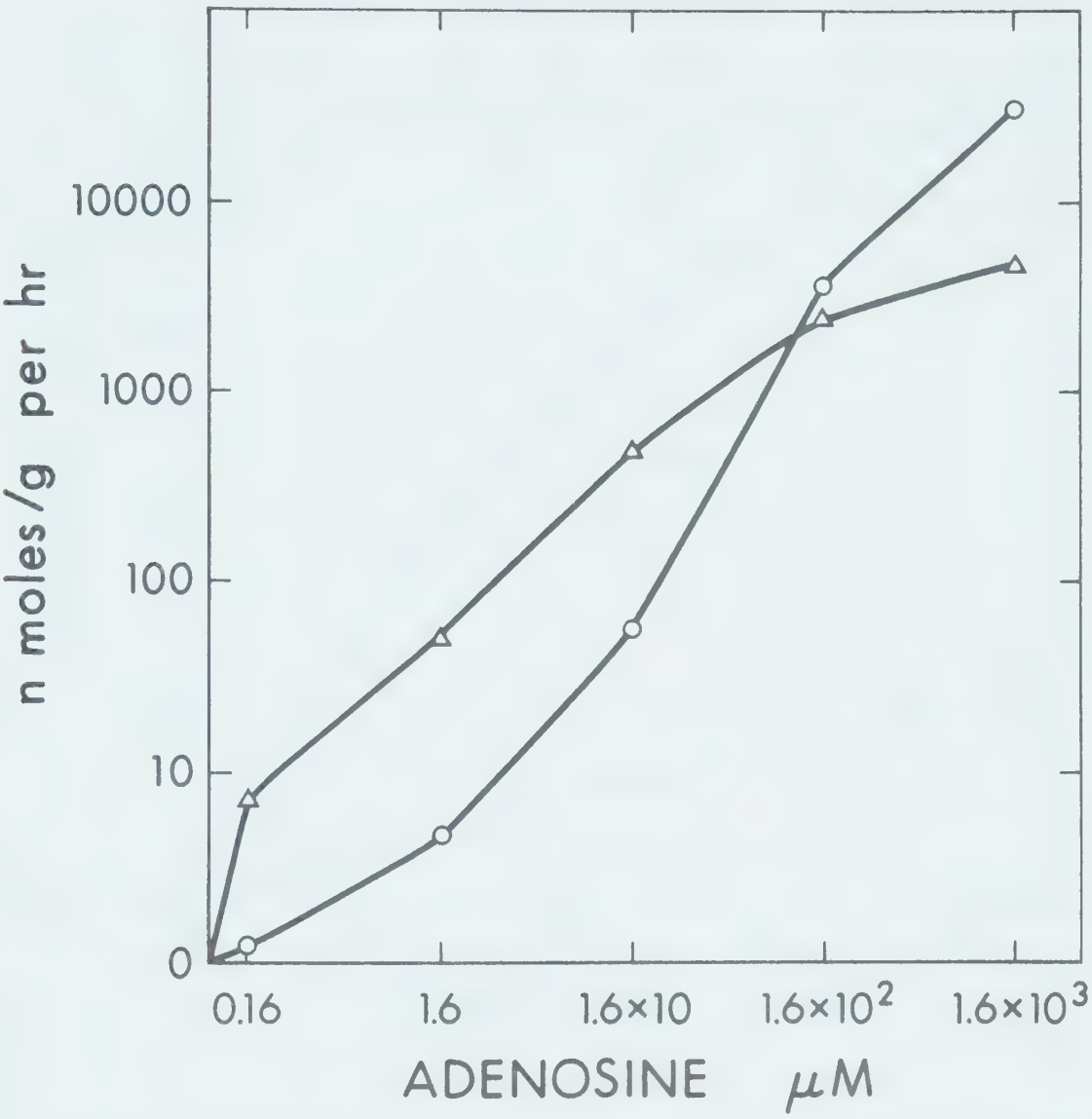


Figure 5. Metabolism of adenosine-^{14}C in Ehrlich ascites tumor cells.

Cells were incubated with adenosine-^{14}C for 60 min after which radioactivity in the products of adenosine kinase, AMP+ADP+ATP (△) and adenosine deaminase, H+HR (o), was measured.

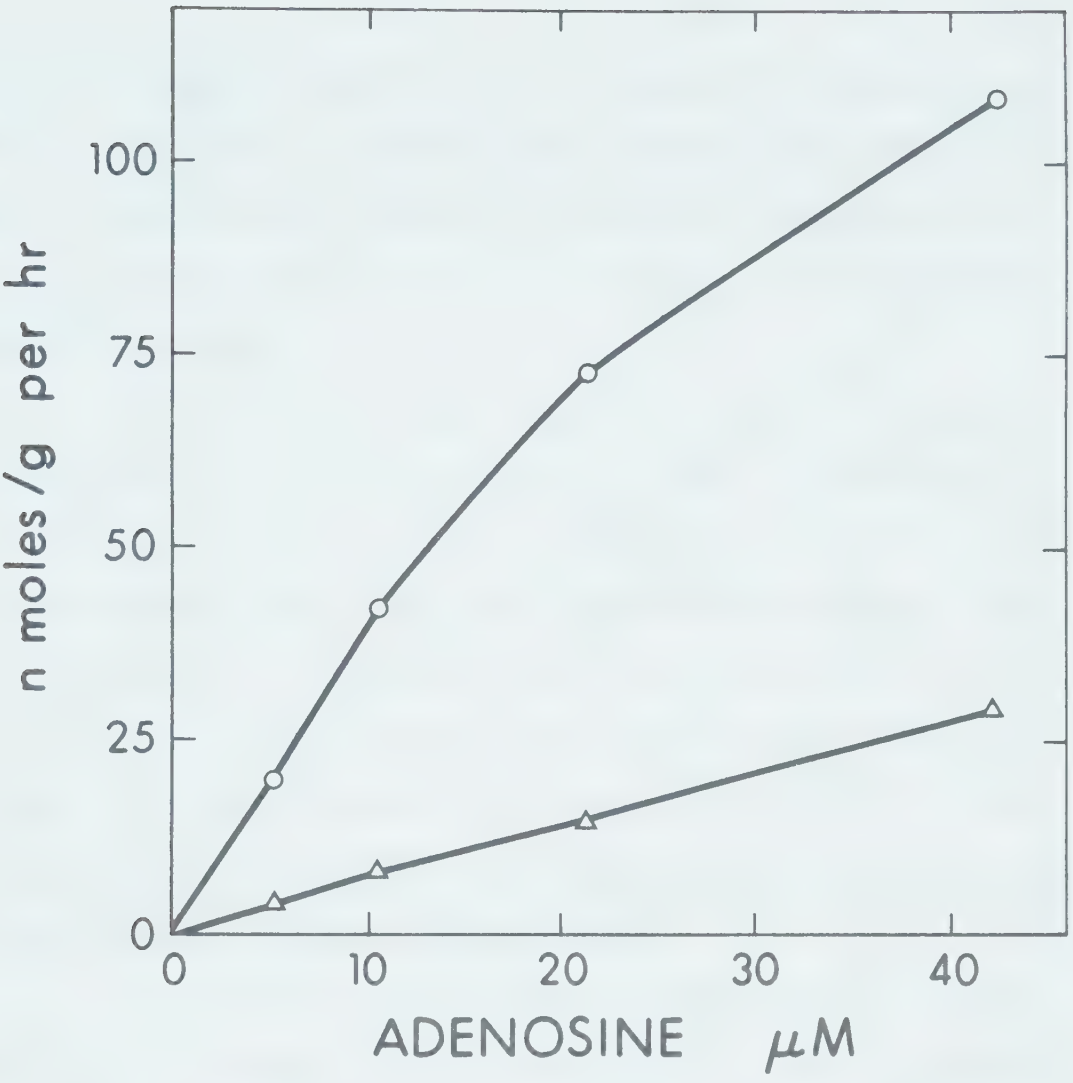


Figure 6. Metabolism of adenosine-^{14}C in sheep erythrocytes.

Cells were incubated with adenosine-^{14}C for 60 min after which radioactivity in the products of adenosine kinase, AMP+ADP+ATP (△), and adenosine deaminase, H+HR (o), was measured.

nucleoside phosphorylase activity measured by determination of the accumulation of radioactive hypoxanthine (Fig. 7). Inosine phosphorylase activity in sheep erythrocytes was found to be approximately 35 fold greater than adenosine deaminase activity.

In view of the low levels of adenosine kinase and deaminase activities observed in sheep erythrocytes, the capacity of these cells to synthesize nucleotides from purine bases was also examined. Nucleotide synthesis from 100 μM adenine-^{14}C, hypoxanthine-^{14}C and guanine-^{14}C was 156, 55 and 6 nmoles/g per 60 min respectively. For comparison, in human erythrocytes nucleotide synthesis from 125 μM adenine-^{14}C and 100 μM hypoxanthine-^{14}C was 355 and 295 nmoles/g per 60 min, respectively.

In view of the evidence presented above for the formation of adenine from deoxyadenosine, several attempts were made to determine if adenine was also formed from adenosine in Ehrlich ascites tumor cells. Cells containing ATP labelled with hypoxanthine-^{14}C were incubated with 5.5 mM 2-deoxyglucose to cause ATP breakdown, 1 μg/ml coformycin to inhibit adenosine deaminase, and 5 mM 2,6-dichloro-9-(tetrahydropyran-2-yl)-9H-purine, which inhibited nucleotide synthesis from adenine by 83%. A small amount of adenine (5 nmoles/g) accumulated after 50 min, suggesting some cleavage of adenosine to adenine. Because of

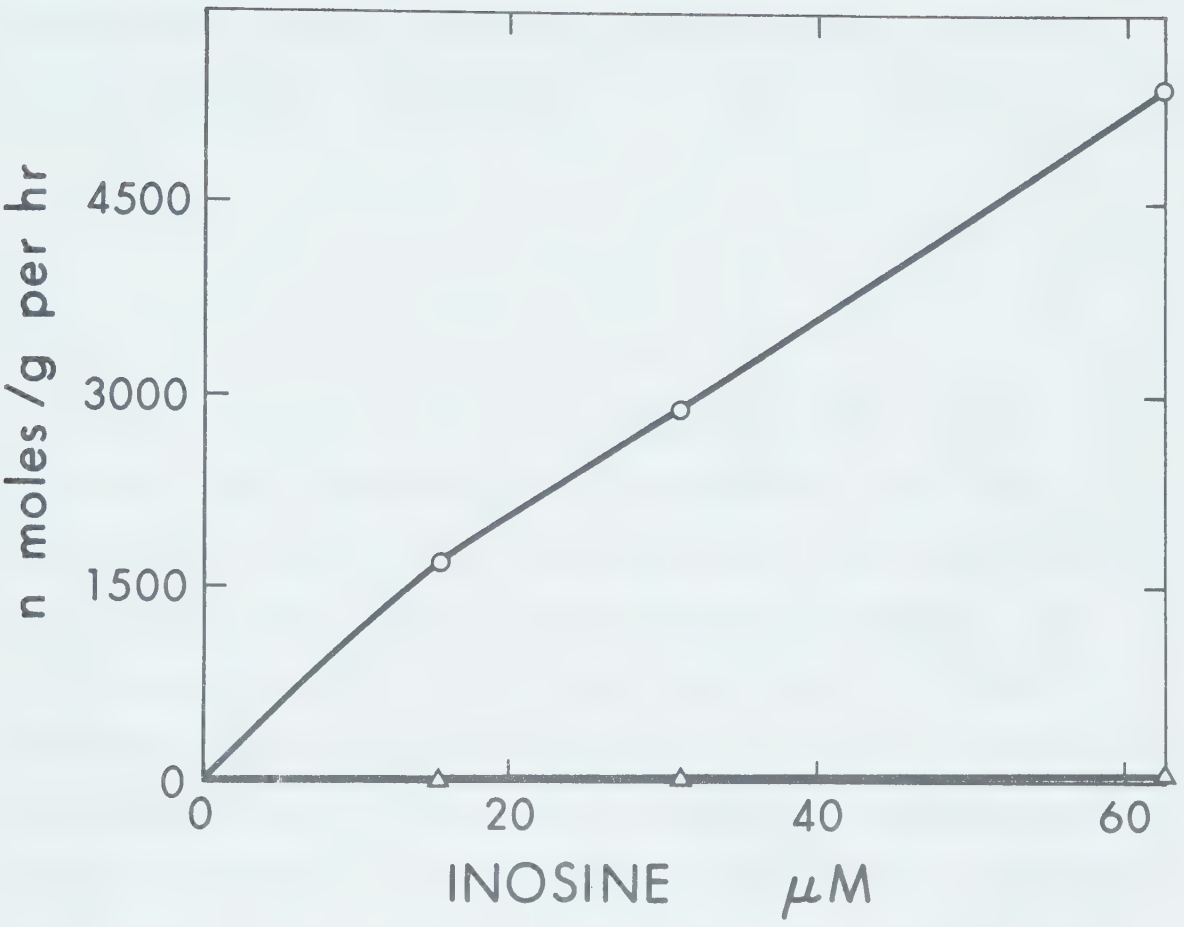


Figure 7. Metabolism of inosine-^{14}C in sheep erythrocytes.

Cells were incubated with inosine-^{14}C for 20 min after which the radioactivity in hypoxanthine (o) and IMP (△) was measured.

the incomplete inhibition of adenine phosphoribosyltransferase activity, however, the exact extent of this process cannot be determined.

DISCUSSION

Alternative routes of deoxyadenosine metabolism have been examined in Ehrlich ascites tumor cells; mouse, human and sheep erythrocytes; and mouse brain, heart, kidney and liver. All of these tissues exhibited a low but measureable ability to cleave the glycosidic bond of deoxyadenosine. Although most previous studies of purine nucleoside phosphorylase from a variety of sources indicated that adenosine and deoxyadenosine were not substrates (12), recent experiments indicate that this enzyme from several mammalian sources can convert adenine to adenosine at low rates in the presence of ribose-1-phosphate (4). Although studies of the metabolism of generally labelled adenosine in rat erythrocytes provided no evidence for phosphorolysis of adenosine, the sensitivity of the methods used was not as great as those used here.

There are but a few previous reports providing information on adenine formation in mammalian cells. Although adenine has not been detected in normal serum (13) or

tissues *in vivo* (14,15), 30 min ischemia (14) leading to the degradation of nucleotides, caused accumulation of adenosine and adenine in rat heart (480 and 80 nmoles/g) and brain (340 and 60 nmoles/g). Adenosine and adenine were also detectable in rabbit kidney and liver under similar conditions (15). Of the four mouse tissues examined in this study, kidney exhibited the highest deoxyadenosine cleaving activity. It is also known that small amounts of adenine, 1.4 mg/day, are excreted in human urine (16). The origin of urinary adenine is unclear, but the widespread distribution of adenine phosphoribosyltransferase (17,18,19) may account for the lack of free adenine in tissues.

Further indirect evidence for a mammalian adenosine cleaving activity has come from studies of H. Ep. #2/MEMPR cells, which have adenine phosphoribosyltransferase but not adenosine kinase activities, and which are resistant to 6-methylmercaptopurine ribonucleoside. Contrary to expectation, they were not cross resistant to 2-fluoroadenosine, suggesting that 2-fluoroadenosine was converted to nucleotide by the sequential action of purine nucleoside phosphorylase and adenine phosphoribosyltransferase (20).

Deoxyadenosine kinase activities were equivalent or up to six-fold higher than deoxyadenosine cleaving activities in tissues incubated in the presence of coformycin

(Table 1). Of the cells examined, erythrocytes had the highest phosphorylation/cleavage ratio. The relative deoxyadenosine cleaving activity (Table 2) was several fold higher in sheep erythrocytes than in human erythrocytes. In contrast to erythrocytes, mouse kidney, brain, liver and heart exhibited near equivalent deoxyadenosine kinase and cleaving activities.

There appears to be some variation in the substrate specificity of adenosine kinases from different tissues. The data of Wong and Henderson (7) indicate that in chopped mouse brain, phosphorylation of adenosine (7) was 70-fold greater than phosphorylation of deoxyadenosine (Fig. 4) at 20 μM nucleoside concentration. Sheep erythrocytes, however, phosphorylated nearly equivalent amounts of deoxyadenosine and adenosine. Deoxyadenosine is phosphorylated by mammalian adenosine kinases from Ehrlich ascites tumor cells and rabbit liver (9), but not by adenosine kinase purified from H. Ep. #2 cells (21) or yeast (22,23). Deoxyadenosine kinase purified from calf thymus used adenosine and guanosine at much lower rates than deoxyadenosine and deoxyguanosine (24). There is indirect evidence that a single enzyme is responsible for the conversion of both nucleosides to nucleoside monophosphates in the Ehrlich ascites tumor cells used in this work; adenosine was phosphorylated approximately 10-fold more rapidly than

deoxyadenosine (5).

A single enzyme is believed to be responsible for the deamination of deoxyadenosine and adenosine in mammalian cells (25,26,27). Because the inosine and deoxyinosine produced by deamination are substrates of purine nucleoside phosphorylase (28), the three potential routes of adenosine metabolism, phosphorylation, deamination and cleavage, are not ordinarily distinguished in studies of intact cells since AMP may ultimately be formed by all of them.

In the absence of coformycin, deamination was the major route of deoxyadenosine metabolism in mouse brain, heart, kidney and liver and in mouse and human erythrocytes, whereas phosphorylation and deamination were nearly equivalent in sheep erythrocytes. Adenosine deaminase activities were nearly equal in sheep erythrocytes and Ehrlich ascites tumor cells at a concentration of 16 μM adenosine, but adenosine kinase activity in Ehrlich ascites tumor cells was 50 times higher than in sheep erythrocytes. Because human and sheep erythrocytes are unable to synthesize adenine nucleotides from hypoxanthine, deamination of adenosine therefore produces a reduction of adenine compounds in these cells. In view of the rapid turnover of adenine nucleotides in erythrocytes (29,30,31), these cells may require a continuous supply of adenine or adenosine to maintain the adenine nucleotide pool.

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CHAPTER 9

VARIATIONS IN PURINE METABOLISM AMONG SUBLINES OF MOUSE TUMORS

INTRODUCTION

Many studies have dealt quantitatively with the kinetics and regulation of enzymes of purine ribonucleotide synthesis and interconversion, and quantitative variations in enzyme activities have proven to be important as bases of metabolic disorders and of drug resistance. In contrast to studies with cell-free preparations, most investigations of enzymes of purine ribonucleotide synthesis and interconversion in intact cells have been only qualitative or descriptive. In this chapter recently developed methods are used to quantitatively measure variations in activities of enzymes of purine metabolism in intact mammalian cells.

MATERIALS AND METHODS

Cells were collected and incubated as previously described (Chapter 2) with 100 μM hypoxanthine-8-^{14}C (49.2 mCi/mmole, Schwarz BioResearch). Procedures for measurement of radioactivity in purine bases, ribonucleosides and ribonucleotides (1), and in acid-insoluble nucleotides (Chapter 2) have been reported. The Ehrlich ascites tumor lines were carried in Ha/ICR mice and mouse lymphoma L5178Y cells were carried in BDF_1 mice.

The metabolism of hypoxanthine-^{14}C was examined in the parent Ehrlich ascites tumor cell line (EAC) and two sublines. Paterson (2) has described the development of a subline, EAC-R1, that is resistant to 6-mercaptopurine (6MP) and which has lost the ability to form 6MP-nucleotide. Another Ehrlich ascites tumor cell line, EAC-R2, was developed (3) that was resistant to 6-methylmercaptopurine ribonucleoside (6MeMPR). The EAC-R2 subline is unable to convert virtually any 6MeMPR to nucleotide (3) and has a reduced ability to convert adenosine and deoxyadenosine to nucleotides (4).

The two L5178Y mouse lymphoma lines examined differed in their sensitivities to the delayed cytotoxic effect of 6MP; the L5178Y-H1 line was less sensitive (D. M. Tidd, personal communication) than the parent line (5). In order to obtain sufficient cells, both lymphoma lines were transplanted to mice and the cells from the first transplant generation were used.

Normalization of apparent enzymatic activities

Methods have been presented for the determination of apparent activities of several enzymes of purine metabolism in whole cells (6). Data for the apparent activity of the first step in the metabolism of a labelled precursor in different cells may be compared directly, provided that

equal concentrations of precursor have been used. Reactions subsequent to the first, have to be corrected for the differing supplies of radioactive substrates that are available to them. In order to facilitate comparison of apparent activities of such reactions in different cells, the following method of normalization has been developed.

The first step in normalizing various sets of experimental results to whichever set of results has been designated as the norm, is the calculation of fractional activities. Fractional enzyme activities are obtained by division of apparent activities measured in one cell line by the corresponding values for the norm. Normalized apparent activities, v^n, are obtained by division of apparent activities of the process being considered, v, by the fractional activity of the preceding process in the metabolic scheme, f^p, so that $v^n = v/f^p$.

For example, consider the conversion of precursor X to Y and Z, in two different cells, E^n and E^a, both incubated with the same initial concentration of X; the activities in experiment E^n are represented by v^n, and those in E^a are represented by v^a.

$$\begin{array}{lccc} E^n & & v_1^n & v_2^n \\ & X & \longrightarrow & Y \longrightarrow Z \\ E^a & & v_1^a & v_2^a \end{array}$$

The apparent activities, v_1^n and v_1^a, may be compared directly. To be able to compare the activities of the enzyme catalyzing the reaction Y$\longrightarrow$ Z, the supply of radioactive substrate Y must be equivalent in both cases. When the activities of cell E^a are normalized to those of E^n, the ratio of supply of Y in the two cell types is given by $v_1^a/v_1^n = f_1$. Thus, if the supply of Y in cell E^a were equal to that in E^n, the normalized apparent activity of the reaction Y$\longrightarrow$ Z would be given by v_2^a/f_1, and this value could then be compared with the apparent activity of this reaction in cell E^n, which is v_2^n.

These methods have been applied to a comparison of hypoxanthine metabolism in Ehrlich ascites tumor cells and sublines EAC-R1 and EAC-R2, and also to mouse lymphoma L5178Y cells and subline L5178Y-H1. Apparent enzyme activities of the parent line have been taken as the norm in each case and normalized apparent activities were calculated for each subline. The metabolism of hypoxanthine-^{14}C (100 μM) was measured over a time course and the normalization procedure was applied to each set of data for a given time period. Values reported for apparent enzymatic activity are averages of duplicate determination.

RESULTS

Ehrlich ascites tumor cells

A comparison of the normalized activities of enzymatic reactions of hypoxanthine metabolism in EAC, EAC-R1, and EAC-R2 cells has been made. For the three Ehrlich ascites tumor cell lines, the normalized activities of the following processes were equivalent and gave near superimposable plots of product formation with respect to time: AMP kinase, ADP kinase, GMP synthetase, GMP kinase, GDP kinase, and GTP ⟶ nucleic acid guanine. Enzymatic activities which showed differences from the parent line are given in Figures 1 to 6. The linear formation of xanthine and uric acid with time (Fig. 1) indicated that 100 μM hypoxanthine was saturating for this process; these data also showed that the EAC-R1 subline has 1.5 times the xanthine oxidase activity of the parent line. IMP dephosphorylase activity (Fig. 2) was reduced in the EAC-R2 line to 0.6 of that in EAC, and the EAC-R1 line had 1.2 fold greater activity than the parent line. The conversion of ATP to nucleic acid adenine (Fig. 3) in EAC-R1 cells was on the average 1.5 fold greater than in EAC or EAC-R2 cells. Differences observed with respect to hypoxanthine phosphoribosyltransferase (Fig. 4), adenylosuccinate synthetase plus lyase (Fig. 5), and IMP dehydrogenase (Fig. 6) were of a lower magnitude.

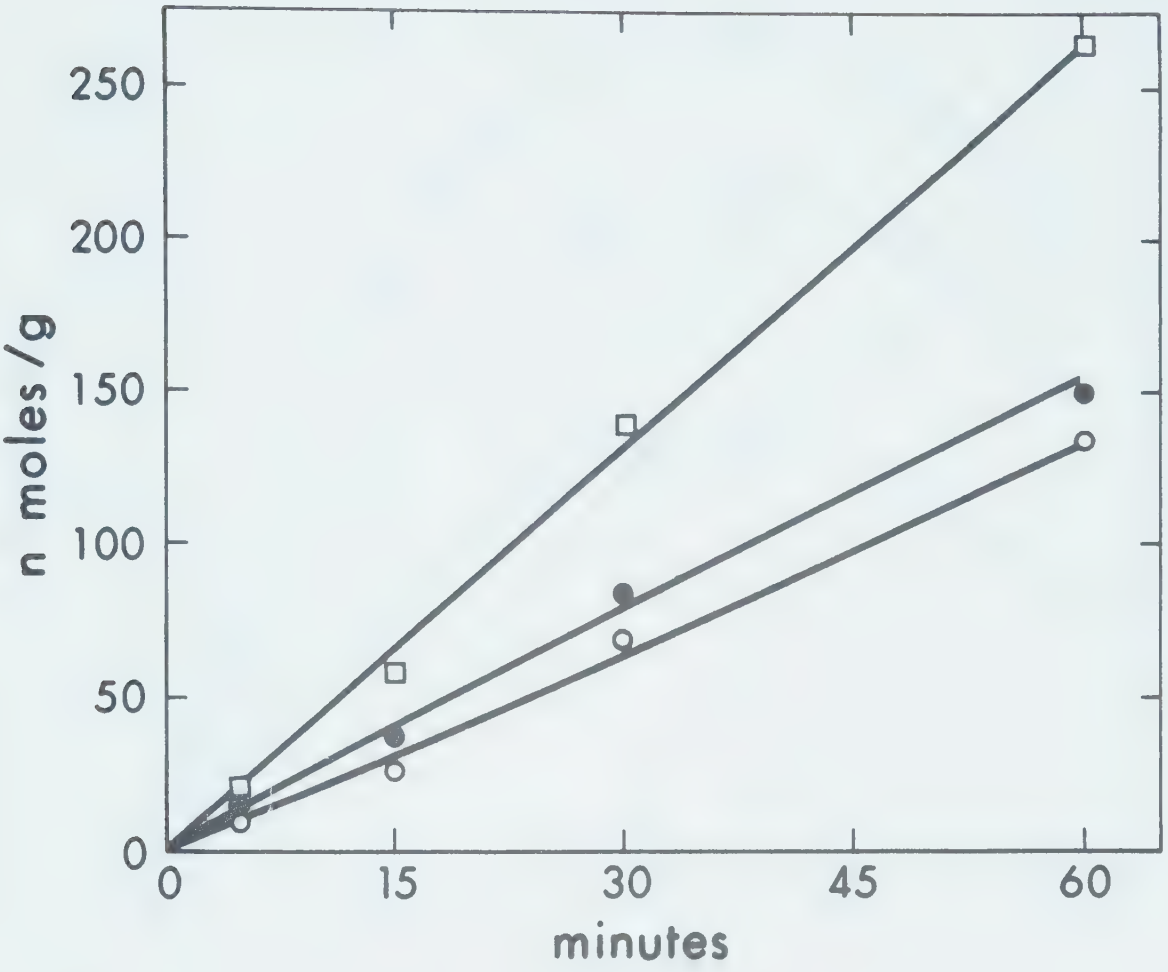

Figure 1. Xanthine oxidase.

(●) EAC
(□) EAC-R1
(o) EAC-R2

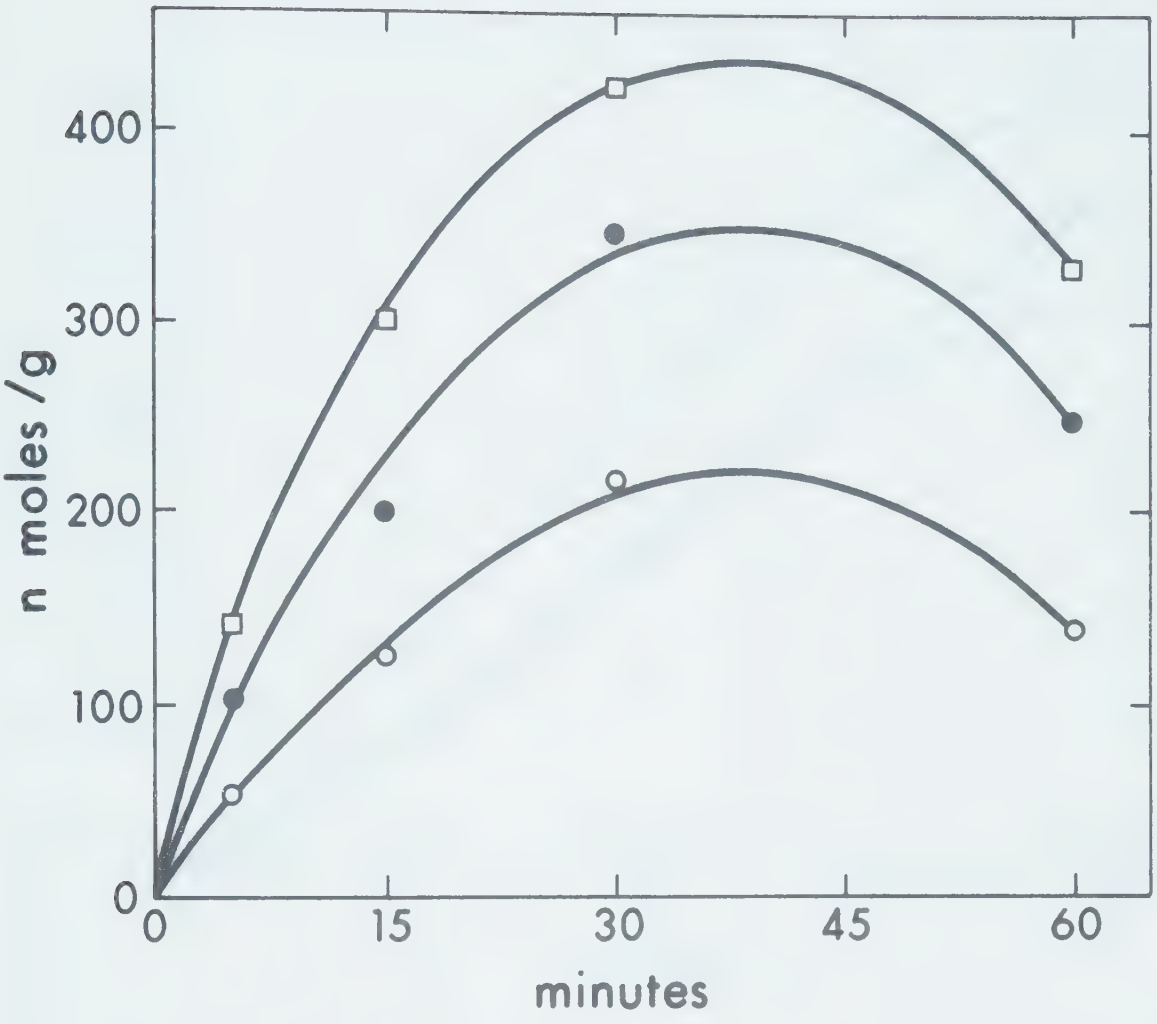

Figure 2. IMP dephosphorylase.

(●) EAC
(□) EAC-R1
(○) EAC-R2

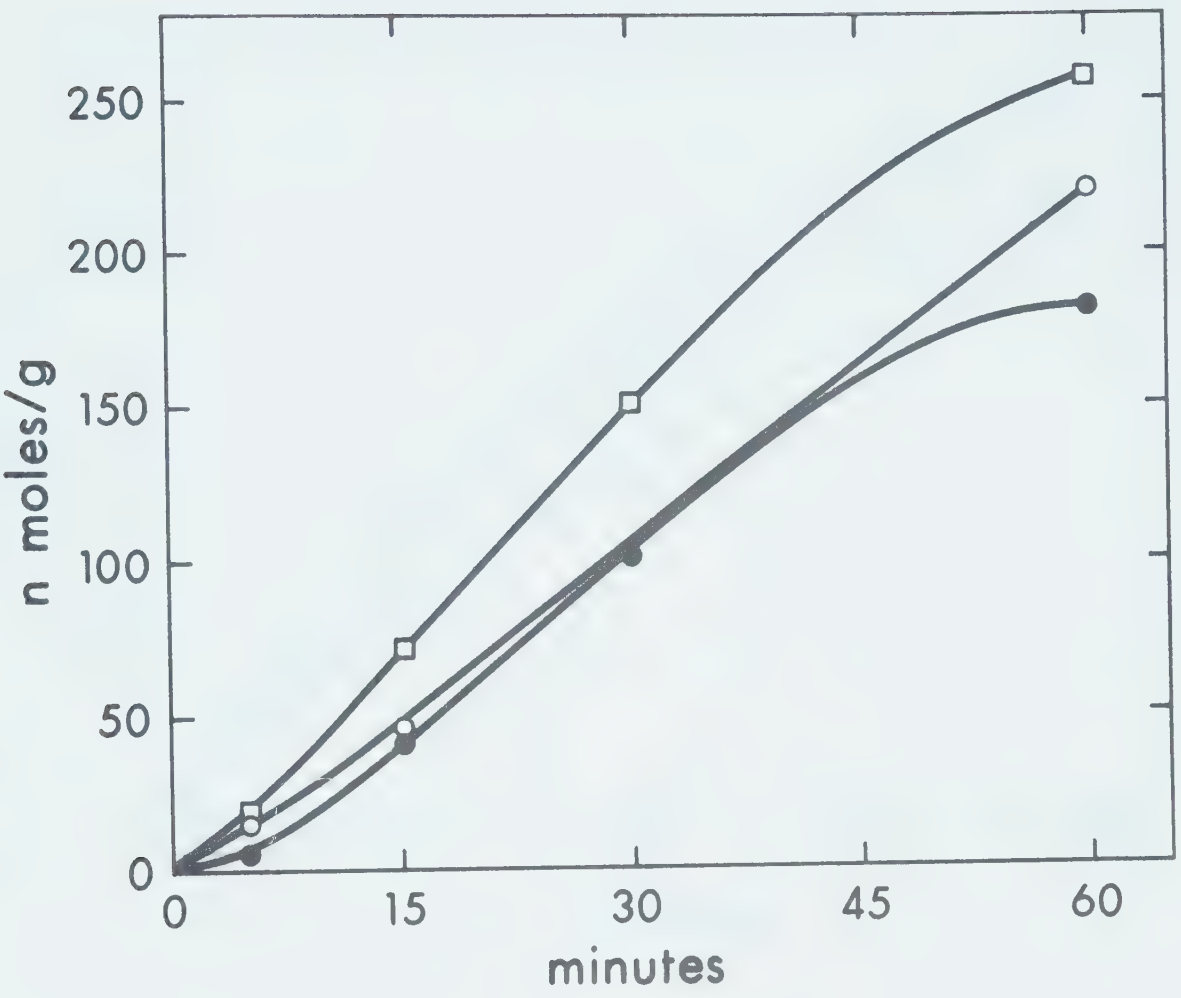

Figure 3. ATP conversion to nucleic acid adenine.

(●) EAC
(□) EAC-R1
(o) EAC-R2

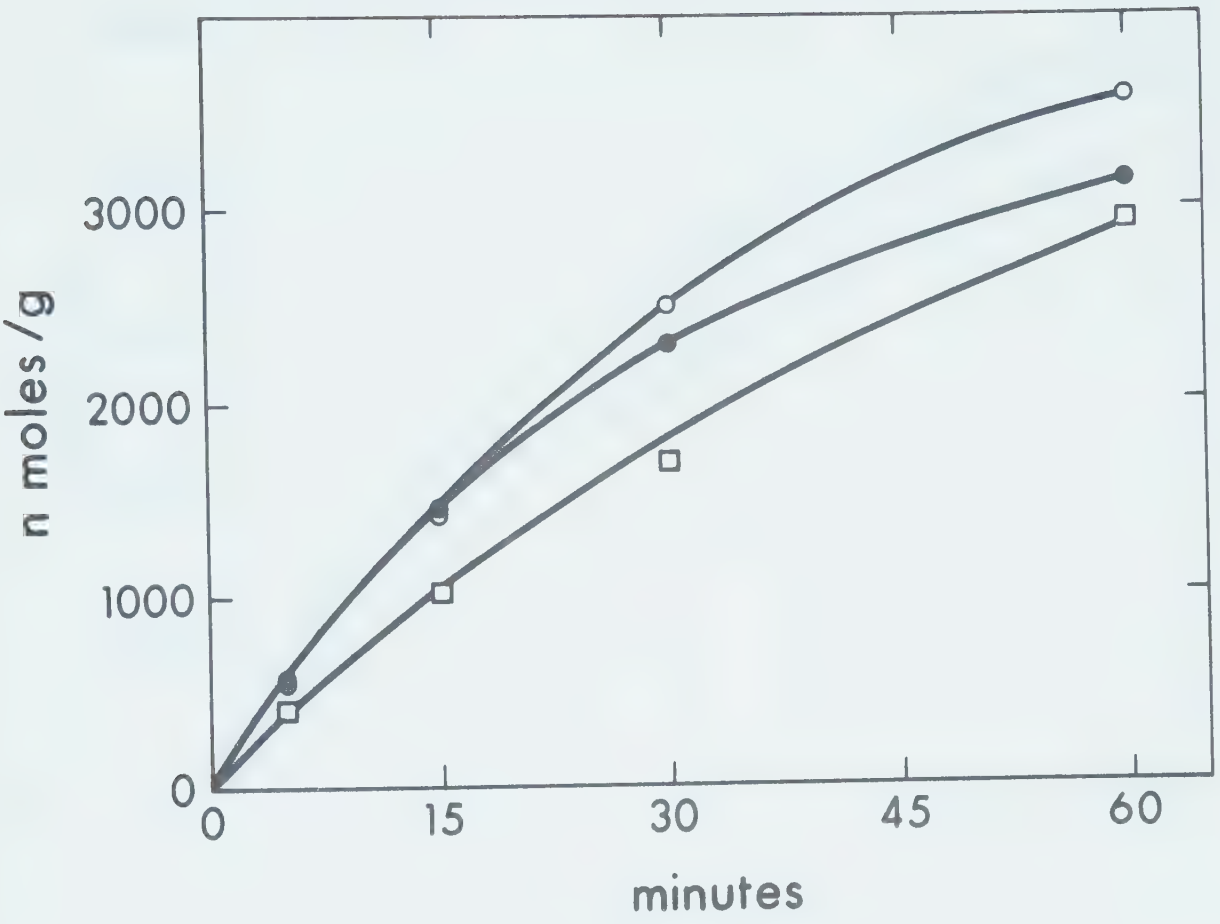

Figure 4. Hypoxanthine phosphoribosyltransferase.

(●) EAC
(□) EAC-R1
(o) EAC-R2

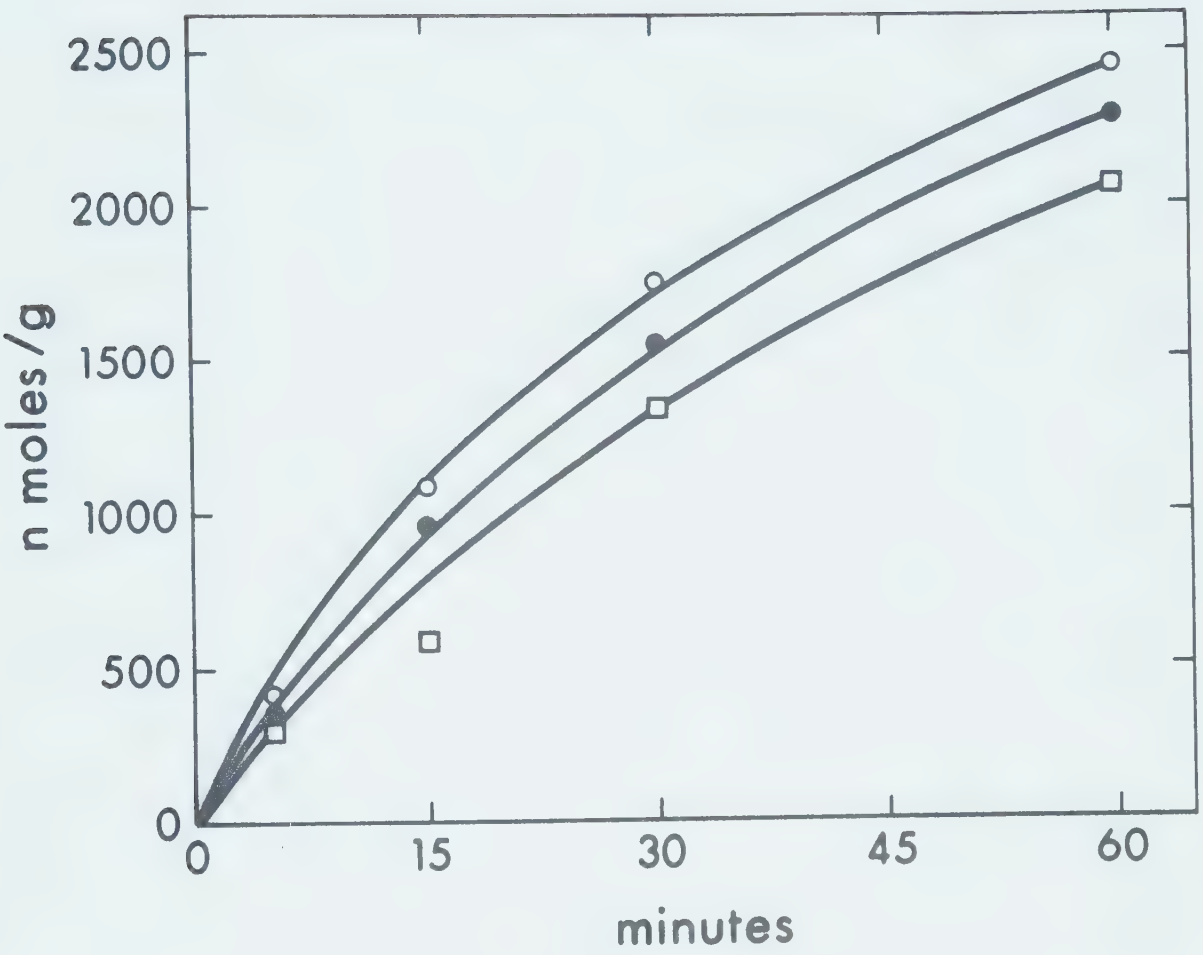

Figure 5. Adenylosuccinate synthetase plus lyase.

(●) EAC
(□) EAC-R1
(o) EAC-R2

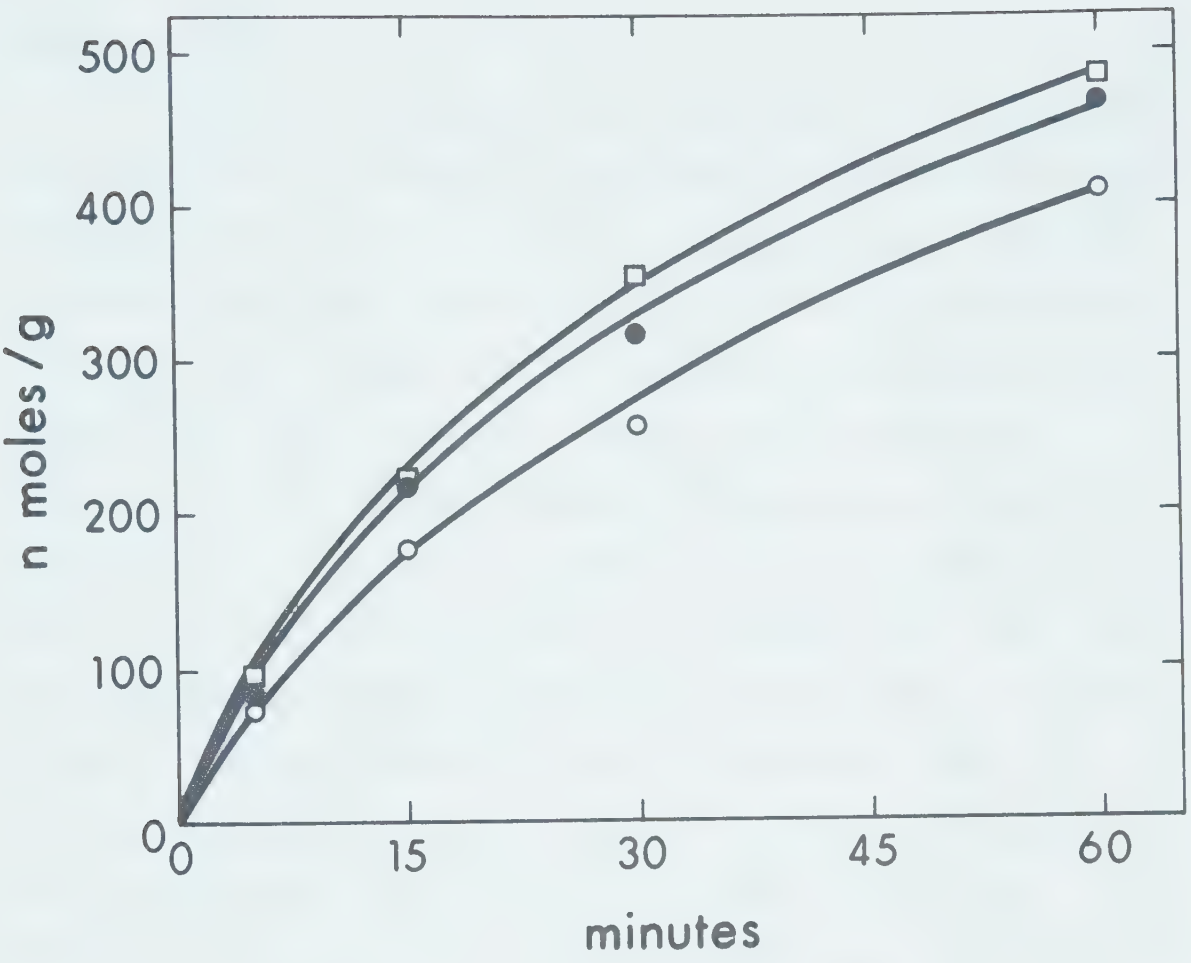

Figure 6. IMP dehydrogenase.

(●) EAC
(□) EAC-R1
(○) EAC-R2

Lymphoma L5178Y

Enzymatic activities in lymphoma L5178Y cells and the normalized activities in the L5178Y-Hl subline have been compared; the following processes had equivalent activities in the two cell lines over the 75 min time course studied: AMP kinase, ADP kinase, ATP ⟶ nucleic acid adenine, GMP kinase, GDP kinase, and GTP ⟶ nucleic acid guanine. Enzymatic activities which showed differences are shown in Figures 7 to 12. Xanthine oxidase activity was two fold higher in L5178Y-Hl cells than in the parent line (Fig. 7), and the activity of IMP dehydrogenase was greater in L5178Y-Hl cells over the initial 30 min of incubation (Fig. 8). IMP dephosphorylase activity (Fig. 9) in L5178Y-Hl cells was approximately half that of the parent L5178Y line. Differences were observed with respect to hypoxanthine phosphoribosyltransferase (Fig. 10) during the initial 30 min of incubation, and with respect to adenylosuccinate synthetase plus lyase (Fig. 11) and GMP synthetase (Fig. 12) during the final 30 min of incubation.

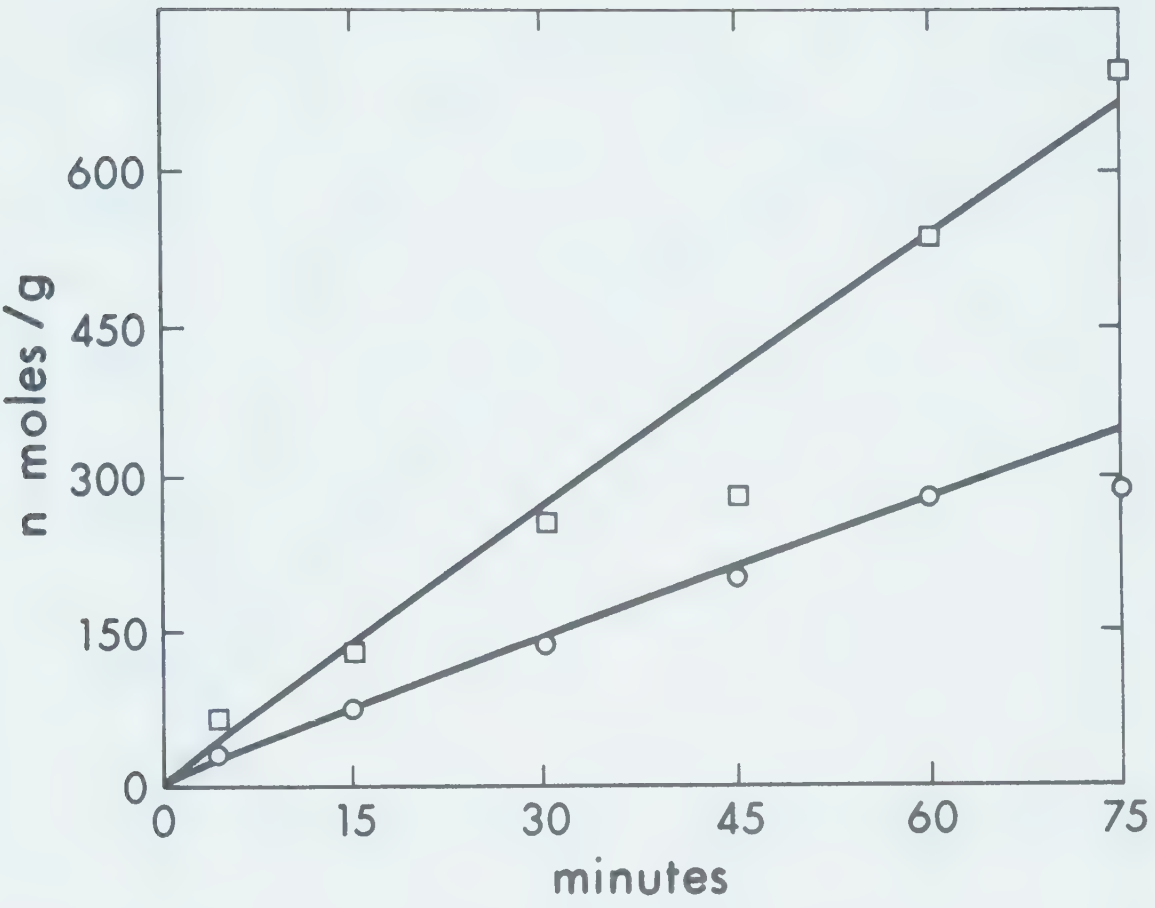

Figure 7. Xanthine oxidase

(o) L5178Y
(□) L5178Y-H1

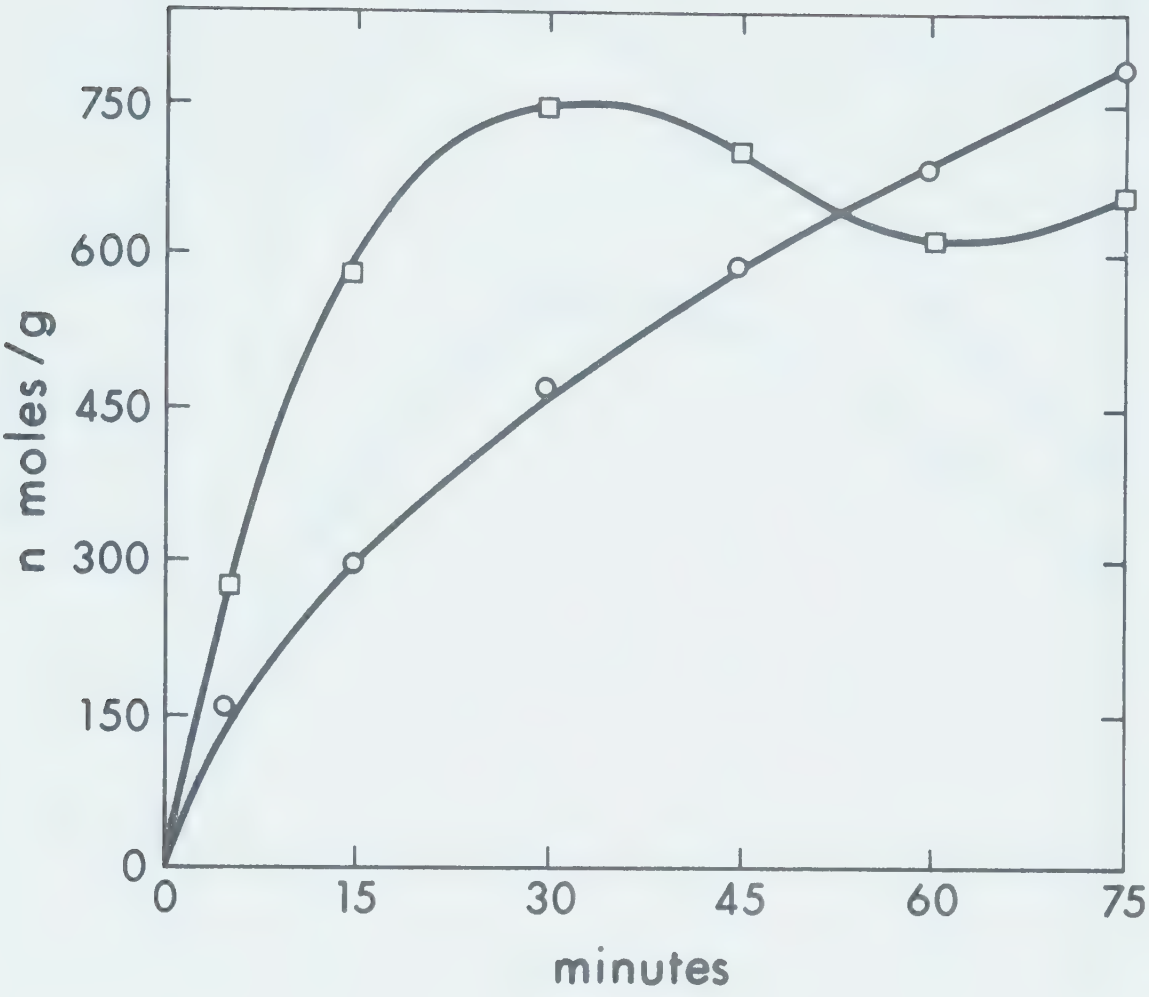

Figure 8. IMP dehydrogenase

(o) L5178Y
(□) L5178Y-H1

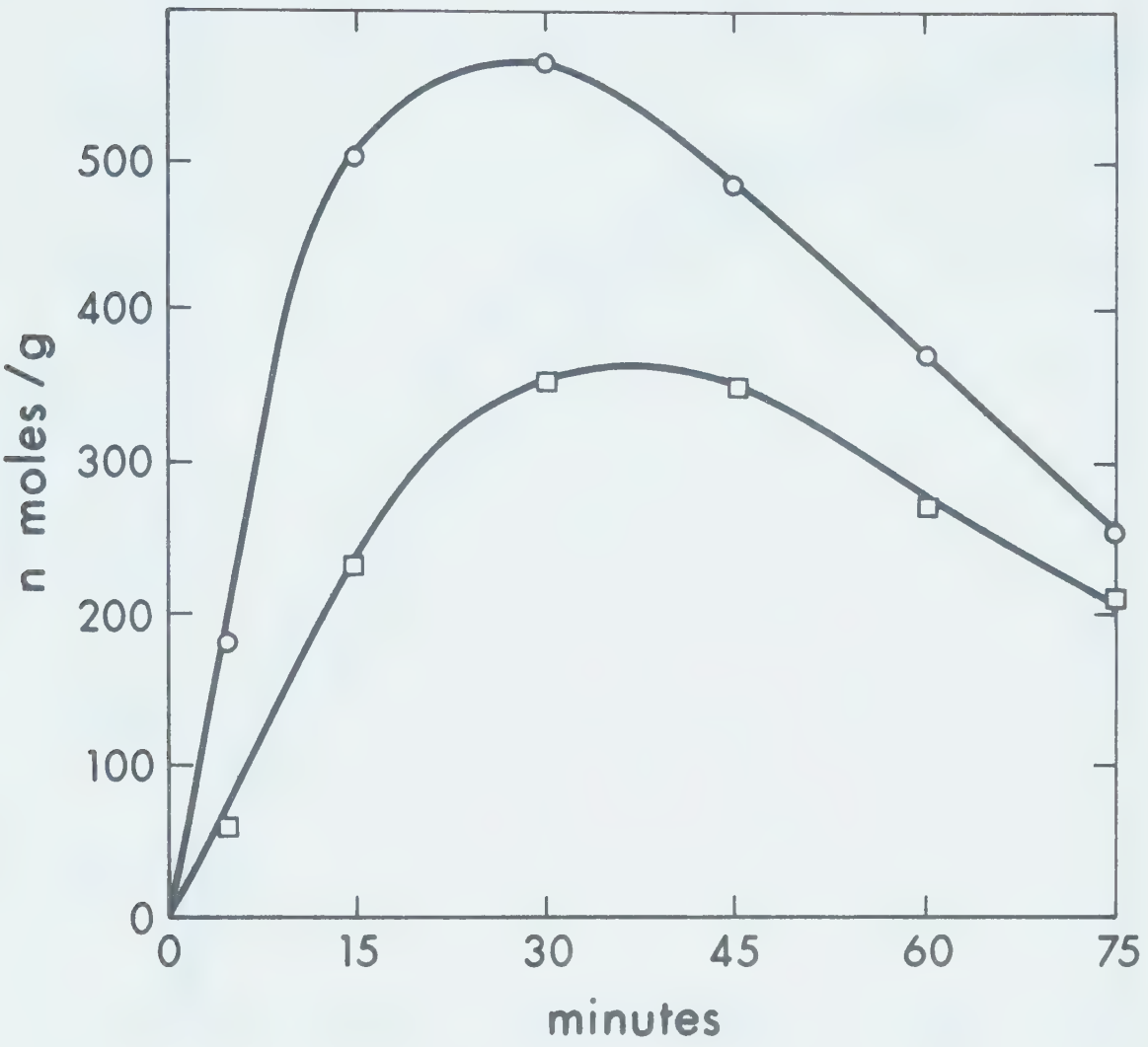

Figure 9. IMP dephosphorylase.

(o) L5178Y
(□) L5178Y-H1

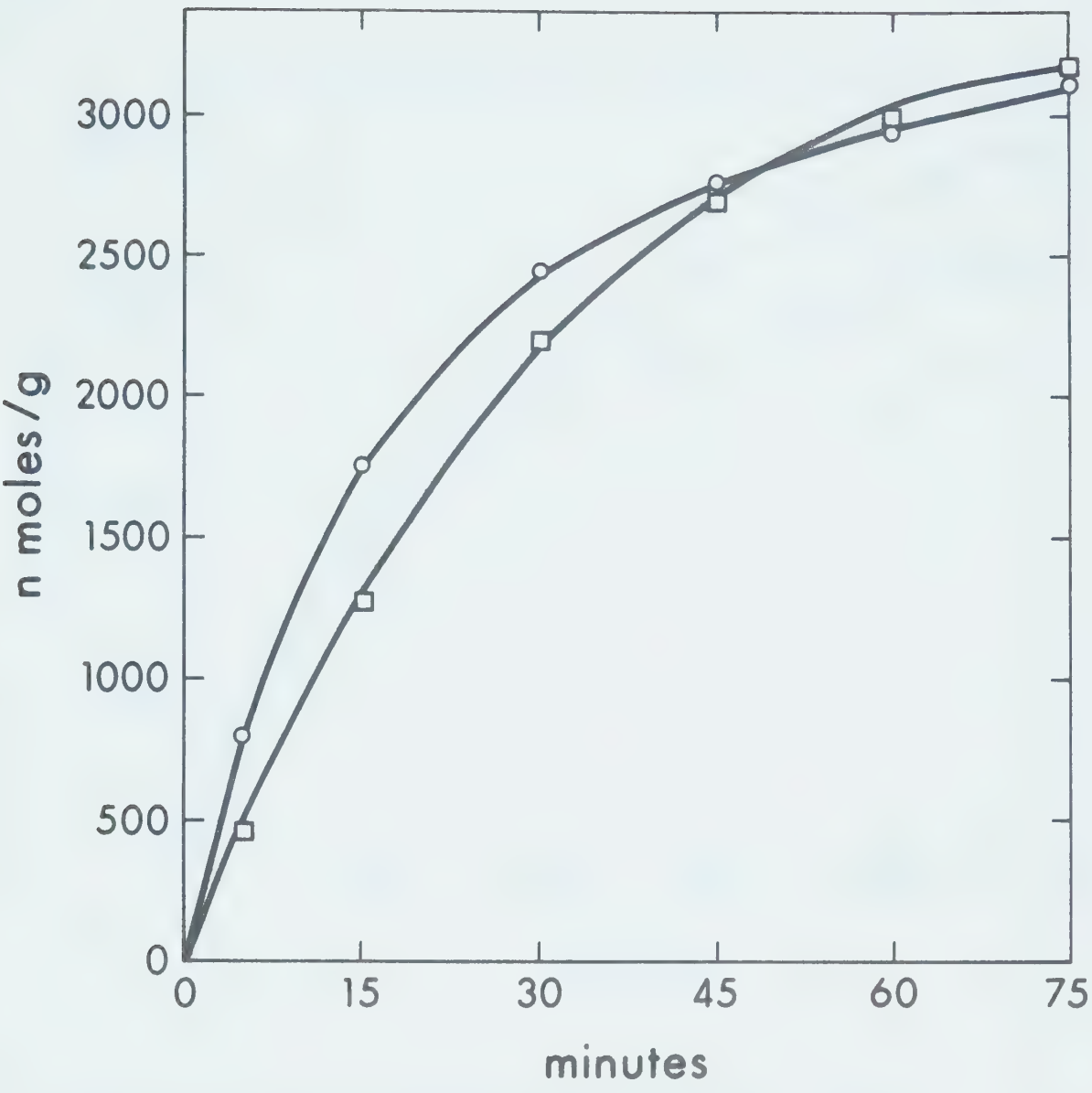

Figure 10. Hypoxanthine phosphoribosyltransferase.

(o) L5178Y
(□) L5178Y-Hl

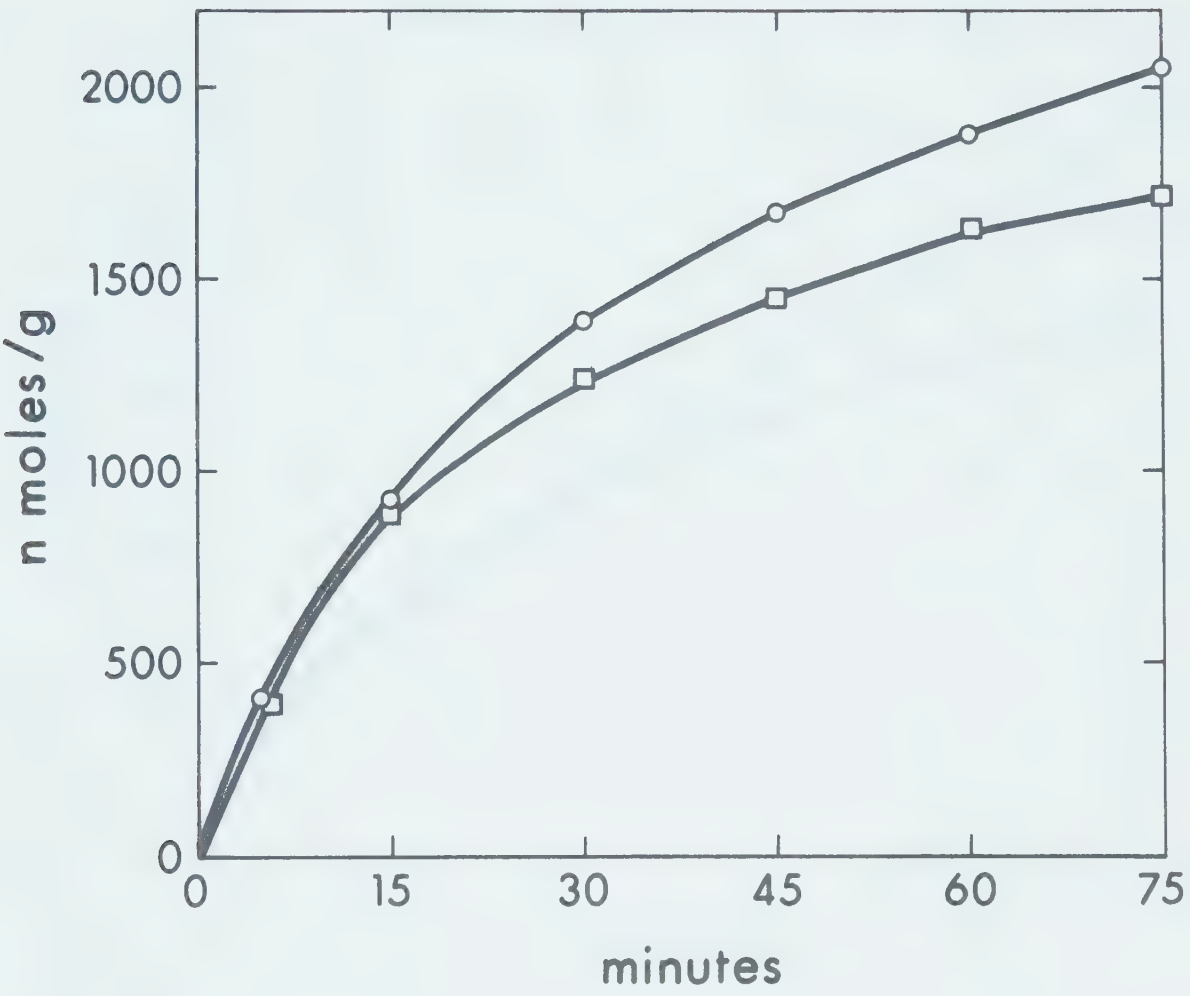

Figure 11. Adenylosuccinate synthetase plus lyase.

(o) L5178Y
(□) L5178Y-H1

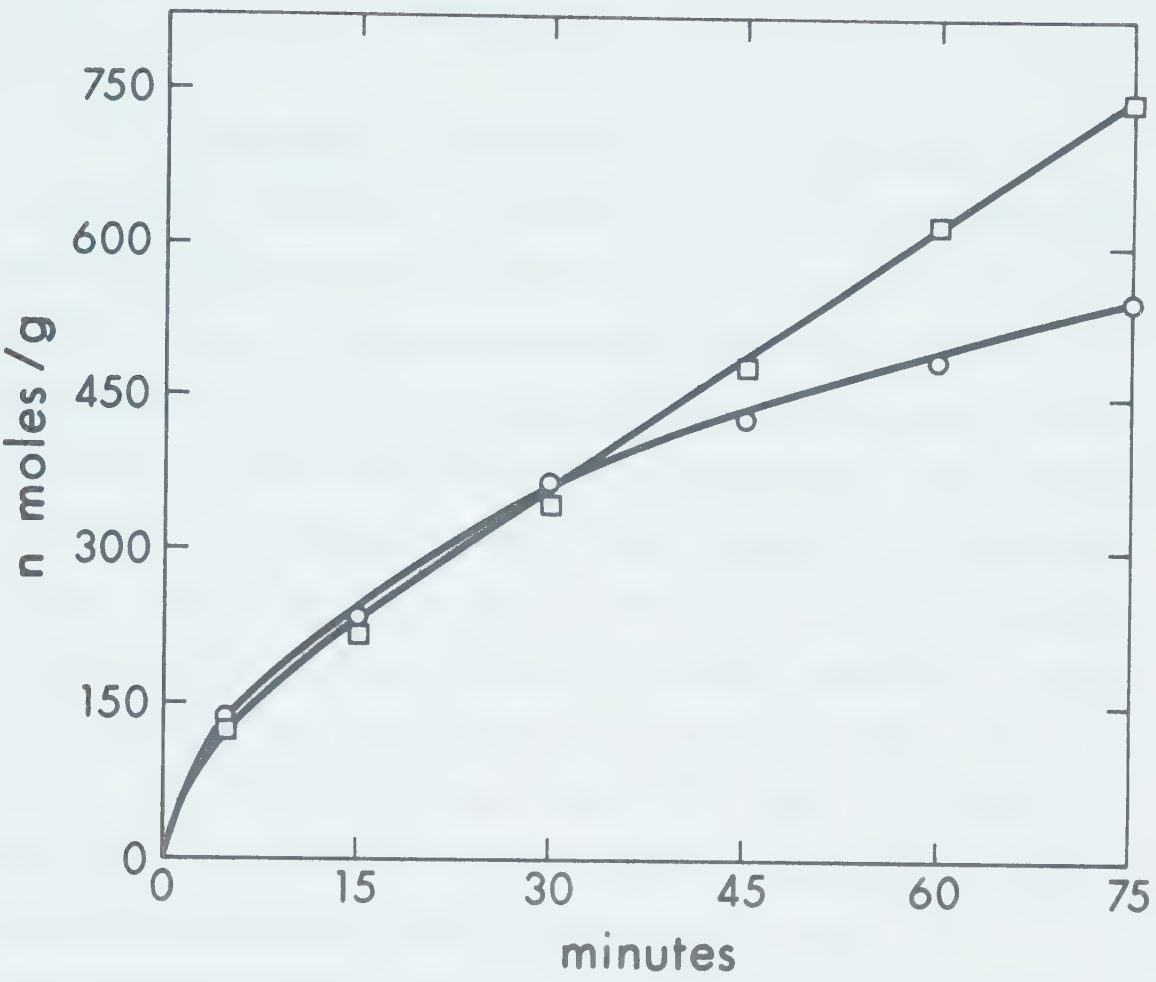

Figure 12. GMP synthetase.

(o) L5178Y
(□) L5178Y-H1

DISCUSSION

This study has detected several differences in the apparent activities of enzymes of purine ribonucleotide synthesis and interconversion in five tumor lines, and this approach therefore appears to have potential as a means of exploring enzymological differences in tumors sensitive and resistant to drugs affecting purine metabolism. For example, the L5178Y-H1 subline, which is less sensitive to the toxic effects of 6MP than the parent line, had a greater xanthine oxidase activity (Fig. 7) than the parent line and a lower rate of conversion of hypoxanthine to nucleotides during the initial 45 min of incubation (Fig. 10); 6MP, of course, is known to be metabolized by both xanthine oxidase (7) and hypoxanthine phosphoribosyltransferase (8,9,10). An accelerated rate of conversion of labelled ATP to nucleic acid adenine in EAC-R1 cells (Fig. 3), may also suggest that the ATP pool in EAC-R1 cells may be smaller than that in EAC or EAC-R2 cells. It is not known for certain however, whether these differences are directly related to the variation in sensitivity of these tumors to 6MP and 6MeMPR.

Although the metabolism of only a single concentration of one labelled precursor over a time course was studied in these experiments, the metabolism of a range of

precursor concentration for a fixed incubation period may also be useful. The use of labelled adenine and guanine, in addition to hypoxanthine, could also broaden the range of enzymatic activities examined. The method of determining normalized apparent activities developed in this work is clearly also applicable to any study which may profit from a comparative analysis, whether variations be due to differences in experimental conditions, cell types, or drug effects.

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